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Preservation of sperm harvested from the rat, caprine, equine, and bovine epididymis

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PRESERVATION OF SPERM HARVESTED FROM THE RAT, CAPRINE, EQUINE
AND BOVINE EPIDIDYMIS

A Dissertation
Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Interdepartmental Program of
Animal and Dairy Sciences

by
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ABSTRACT

The interest in preserving endangered species has increased the amount of attention lent to the recovery of functional sperm from the epididymides of deceased males (Foote, 2000). Postmortem specimens have a finite time period before decomposition affects functionality. Determination of this “window of opportunity” to harvest and preserve epididymal sperm would be beneficial for further research in sperm preservation and assisted reproductive technologies. The objective of these studies was to determine 1) the “window of opportunity” to collect viable rat, caprine, equine and bovine epididymal sperm, 2) if epididymal sperm collected could be cryopreserved, 3) to test two common cryoprotectants for efficacy of sperm preservation, 4) to determine if bovine samples could be used to produce in vitro fertilization (IVF) embryos and 5) to establish if sperm subjected to a series of freeze-thaw cycles can maintain motility. Epididymal sperm collected from rat, caprine, equine and bovine males all maintained some level of acrosomal membrane integrity up to 96 hours postmortem. The bovine, caprine and equine sperm survived cryopreservation and exhibited greater preservation with milk-based extenders. In vitro fertilization with cryopreserved bovine epididymal sperm was not efficient but development of embryos proved limited usefulness. Finally, subjecting the bovine sperm to repeated freeze-thaw cycles proved extremely damaging and should be practiced only when absolutely necessary.

Rat sperm exhibited a difference from 24 to 48 hour with a percent progressive motility (PPM) of 46 to 28%. Caprine sperm PPM and percent intact acrosomes (PIA) declined after 24 hours from 68 and 66% to 56 and 55% at 48 hours, respectively. Equine sperm exhibited a drop in PPM and PIA at 48 hour of 42 and 71% to 34 and 68% at 72 hours, respectively. Bovine sperm PPM dropped initially from 65 to 49% at 24 to 48 hours

and again from 46 to 30% at 72 to 96 hours. The difference in PIA only appears between 24 and 48 hours of 77 to 65%. As stated previously, the epididymal sperm collected from the rat, caprine, equine and bovine males maintained acceptable levels of PPM and PIA up to 96 hours postmortem.

INTRODUCTION

Genome banking (cryopreservation) has become an important management strategy for the conservation of endangered and/or exotic species (Loskutoff et al., 1996). The effort to conserve embryos and sperm from exotic species is a relatively new management technique used to help maintain genetic variation needed to maintain species viability. Loskutoff and Betteridge (1992) have reviewed cases involving assisted reproductive technologies for preserving nondomestic species. Even the advances made thus far, much research is still needed, especially in the preservation of semen from nondomestic species, both ejaculated and epididymal sperm (Loskutoff et al., 1996). The interest in preserving endangered species and valuable genetic material has resulted in increased attention to the possible recovery of viable sperm from the epididymides of dead animals (Foote, 2000).

In a letter to the editor of the Journal of Andrology, Foote (2000) discussed the increasing interest in preserving functional sperm from deceased animals of exotic and/or endangered species. Those groups that are working to preserve endangered species have become increasingly interested in this area because of new management techniques for conserving the genetic diversity of diminishing animal populations. It was indicated in this letter that research on preservation of epididymal sperm from dead animals has been mostly in the mouse (Songsasen et al, 1998; An et al., 1999a, 1999b). During years of teaching comparative reproductive physiology, Foote (2000) found that he and other professors could obtain motile sperm from the epididymides of bulls, boars, rams and stallions at 24 to 48 hours after death. It is also interesting to note Vishwanath and Shannon (2000) describe the use of storing liquid semen at 5°C to increase the longevity of sperm. Bovine semen was successfully stored at 5°C for more than 4 days. Previous

research has given encouraging evidence that further investigations should be made into the duration of sperm functionality maintained by liquid cool storage.

It is important to learn how to maintain sperm viability within the epididymides after death because researchers doing field work with endangered species usually do not have access to a laboratory. In such cases, it would be beneficial if the testicles and/or the epididymides could be harvested and packaged to maintain at least 5°C, then transported to a laboratory where the epididymides could be properly processed for sperm recovery. This requires that functional sperm be maintained for several days or more. Since gametes of endangered or exotic species are very valuable, research should be completed using domestic animals to determine the basic protocols. Once determined then research can be conducted to modify the protocols to best fit the endangered species being studied.

CHAPTER 1

REVIEW OF LITERATURE

Epididymal Sperm

To study epididymal sperm it becomes necessary to first understand physiological process of its formation as well as that of the final product, ejaculated sperm, so that the best methods for working with epididymal sperm can be established. The objective of the following section is to review important physiological parameters of epididymal sperm.

Maturation of the epididymal sperm membrane has been referred to as membrane remodeling. As sperm pass through the epididymis there is extensive “remodeling” of the surface carbohydrates (Dacheux and Voglmayr, 1983; Jones et al., 1983; Young et al., 1985; Parillo et al., 1997; Jones, 1998; Kirchhoff and Schroter, 2001). Modifications to the sperm surface may result from changes in removal of, or unmasking of pre-existing components, or the adsorption of macromolecules produced by the epididymis (Dacheux and Voglmayr, 1983; Young et al., 1985; Kirchhoff and Schroter, 2001).

The physiological effects of epididymal acquisition of macromolecules are not understood but they may be required for the development of sperm motility and fertilizing capacity (Young et al., 1985; Jones, 1998). Studies in the rat (Blandau and Rumery, 1964), hamster (Horan and Bedford, 1972), guinea pig (Young, 1931), rabbit (Nishikawa and Waide, 1952; Paufler and Foote, 1968; Orgebin-Crist, 1969) and pig (Holtz and Smidt, 1976) reported higher fertilization or pregnancy rates with epididymal sperm collected from the cauda epididymidis as compared with samples collected from more proximal regions. Kumar et al. (1990) reported that epididymal maturation involved a reduction in phosphatidylcholine (PC) (lecithin) and cholesterol as well as the proportion of saturated to

unsaturated fatty acids. These particular changes are directly involved with membrane structure and reduce the stability and increase membrane fluidity.

Epididymal sperm maturation greatly affects membrane lipid composition. There are three unusual features of sperm lipids, 1) the presence of large amounts of plasmalogen phospholipids up to 40%, 2) the high content of polyunsaturated fatty acids and 3) relatively low cholesterol:phospholipid ratios that are species specific (Jones, 1998). During passage through the epididymis, there is a 25 to 48% decrease in the total cellular content of phospholipids. There is relatively little change in the amount of choline plasmalogen (CP) while the other phospholipids undergo major concentration changes, indicating that changes during maturation are very specific (Jones, 1998).

Based on this understanding, what factors mediate selective phospholipid loss and what are the implications for the properties and behavior of the plasma membrane? In epididymal fluids glucose is low or nonexistent, which is likely the reason that fatty acids from the phospholipids might provide an energy source for maturing sperm (Mann, 1964). This premise is supported by the presence of high concentrations of carnitine and acetylcarnitine that aid in the transfer of fatty acids into mitochondria for β -oxidation (Deana et al., 1989).

Membrane remodeling of the sperm surface also affects the protein components. The most obvious and best-documented path for membrane remodeling is the uptake of glycoproteins directly from epididymal luminal secretions (Dacheux and Voglymayr, 1983; Young et al., 1985; Kirchhoff and Schroter, 2001). Approximately 50% of the added glycoproteins are synthesized in specific regions and bind to the sperm cell as they pass through the lumen. Another method for remodeling is the processing of acquired or

pre-existing glycoproteins by glycoprotein modifying enzymes (Tulsiani et al., 1998) and migration of antigens to new membrane domains (Jones, 1998).

Acetyl carnitine (γ -amino- β -hydroxybutyric acid trimethylbetaine) is known to facilitate mitochondrial β -oxidation of fatty acids and is present at the highest levels in epididymal fluid. This role of oxygen consumption has been confirmed in rat, rabbit and bull sperm mitochondria but it has also been shown to inhibit respiration of ejaculated bovine sperm (Deana et al., 1989). It enhances maintenance of membrane integrity and viability of cells subjected to adverse conditions (Snyder et al., 1990; Arduini et al., 1997).

Deana et al. (1989) described some effects of carnitine on functions of ejaculated bovine sperm such as motility, oxygen consumption, calcium transport and release of enzymes. L-carnitine activates calcium cycling in bovine sperm (Deana et al., 1989). Calcium controls several metabolic processes of sperm, namely motility and oxygen consumption (Deana et al., 1989). Ejaculated sperm have a much lower capability for transporting calcium than epididymal sperm (Deana et al., 1989). This is probably due to a seminal plasma protein (caltrin) that binds strongly to the plasma membrane, preventing calcium uptake by ejaculated sperm (San Agustin et al., 1987). Morris et al. (2000) also found a difference in the calcium distribution of equine epididymal sperm compared with ejaculated sperm. A higher calcium uptake in response to carnitine would be expected for epididymal sperm. Deana et al. (1989) have suggested that the role of L-carnitine in epididymal fluid is to maintain quiescence of which the mechanism is unknown, however, it was thought to be due to inhibition by the tricarboxylic acid (TCA) cycle.

Epididymal sperm use different substrates as energy sources, but fatty acid oxidation, involving the carnitine-dependent systems, seems to be the major source of energy (Casillas, 1972). By contrast, cellular and metabolic functions of ejaculated sperm

depend more on glycolysis and oxidation of pyruvate (Storey and Keyhani, 1974; Hutson et al., 1977).

Ejaculated Sperm

This section will briefly address some differences in the sperm cell biology after ejaculation. The phospholipid, fatty acid and cholesterol composition of the sperm surface membrane changes after ejaculation. Phospholipids and cholesterol are important membrane components especially when concerned with cold shock, which causes the membrane to lose lipids. Sperm susceptibility to cold shock has been correlated to a high ratio of unsaturated:saturated fatty acid content as well as a low cholesterol content of the outer sperm membrane (White, 1993). Bull and ram sperm from the rete testis or the caput epididymidis are more tolerant of cold shock than ejaculated sperm (Voglmayr and Sawyer, 1986; Windsor et al., 1988; White, 1993). Thus, sperm during maturation develop a higher sensitivity to cold shock. In the ram, sensitivity develops first in the proximal corpus epididymidis where lower molecular weight glycoproteins disappear from the sperm surface (Voglmayr and Sawyer, 1986; Windsor et al., 1988).

Fatty acid composition of most mammalian sperm is as follows: the principal phospholipid-bound unsaturated (containing a double bond) fatty acid is docosapentaenoic (22:5) or docosahexaenoic (22:6), while the major saturated fatty acid is palmitic (16:0) (White, 1993). The ratio of unsaturated to saturated fatty acids in the phospholipids of sperm fall into two groups; a high group with bull, ram, and boar sperm exhibiting a ratio of 2.5:3.0 and a low group with rabbit, dog, human and fowl sperm having a ratio of 1.0:1.0. This ratio grouping coincides with their sensitivity to cold shock, with the high ratio group showing greater sensitivity. White (1993) also stated that the proportion of unsaturated fatty acids might have a significant effect on properties of the sperm

membranes, including maintenance of the correct “fluidity” at standard environmental temperatures and nonelectrolyte permeability as well as the temperature at which phase change occurs in membrane phospholipids.

Membrane models show that polyunsaturated fatty acids form expanded films and in the presence of cholesterol the fatty acids will condense (White, 1993). This could be an important role for cholesterol in that it would aid in the formation of an impermeable and cohesive membrane structure when large amounts of unsaturated fatty acids are present. Cholesterol content of ram and bull ejaculated sperm is approximately $300 \mu\text{g}/10^{-9}$ sperm, while rabbit and human sperm contain approximately $550 \mu\text{g}/10^{-9}$ sperm, corresponding to the more susceptible and less susceptible to cold shock groups, respectively (White, 1993).

Leboeuf et al. (2000) indicate two components of caprine seminal plasma that when combined with either egg-yolk or milk-based diluents are detrimental to motility and/or viability. It was noted that the first component is egg-yolk coagulating enzyme (EYCE, identified as a phospholipase A) is produced by the bulbourethral gland. When mixed with egg-yolk diluents, caprine sperm viability was impaired. Phospholipase A catalyses the hydrolysis of egg-yolk lecithin into fatty acids and lysolecithin, and the lysolecithin was found to be toxic to caprine sperm.

The second component is also generated in the bulbourethral gland and is a protein identified as a monomeric 55- to 60-kDa N-glycosyl-protein (BUSgp60) with heparin affinity (Leboeuf et al., 2000). This protein displays triacylglycerol hydrolase activity. Addition of milk-based medium to caprine semen creates an interaction with BUSgp60 to inhibit sperm motility. The detrimental component in the milk-based medium that was interacting with BUSgp60 was found to be oleic acid hydrolyzed from milk triglycerides. Washed ejaculated buck sperm after cryopreservation was shown to have the same quality

as cryopreserved epididymal sperm, indicating that the detrimental components are present in the seminal plasma.

Methods of Epididymal Sperm Collection

There are a variety of ways to collect epididymal sperm and both from dead or live sperm donors. Collection from deceased donors is easier because the entire testicle can be easily removed for processing; whereas, live donors must be restrained, either by chemical or physical means. The goal is usually to obtain live sperm to use with assisted reproductive technologies such as artificial insemination (AI), in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) for production of offspring.

Rat, porcine and bovine caput and caudal epididymal sperm have been collected postmortem for experimental purposes by tissue mincing and incubation in various media (Vierula and Rajaniemi, 1982; Bawa et al., 1993; Tulsiani et al., 1993). Epididymal sperm has been extracted from ram (Dacheux and Voglmayr, 1983) and rat (Jones et al., 1983) testicles after surgical removal and the epididymis was microperfused to obtain the sperm. This method prevents contamination of sperm with extraneous cellular material and fluid components. Epididymal sperm has been collected from marmoset monkeys by extracting the cauda epididymidis and vas deferens, piercing the lumen with a 20-gauge needle and gently washing the tissue with medium (Morrell et al., 1997).

A method for microsurgical epididymal sperm aspiration (MESA) in men was described by Monseny et al. (1994). A small incision was made through the scrotum to expose the epididymis and a single epididymal tubular loop was freed under the surgical microscope. Next a longitudinal incision was made into the epididymal tubule. To aspirate sperm, a 0.2-mm diameter, modified glass capillary attached by a silicon tube to a reservoir containing the culture medium is connected to another tube connected to a 50-ml

syringe to create negative pressure is inserted into the tubular lumen. After completion then the tubule can either be closed by stitching or left open for further aspirations, transscrotally (Moni, 1992).

Monseny et al. (1994) also described a technique for sperm aspiration from the vas deferens in men called microsurgical deferential sperm aspiration (MDSA). With this procedure a transversal hemi-section is made to visualize the lumen. A longitudinal incision is made in a vas deferens tubule and an aspirating device is inserted into the proximal end of the lumen. Fluid is aspirated by applying gentle digital pressure to the testicle and epididymis. Once completed, the tubule incision is closed with two to three stitches followed by closure of the scrotum.

Another method of human epididymal sperm collection from live donors who cannot produce an ejaculate is percutaneous epididymal sperm aspiration (PESA). This method is considered nonsurgical because no large incisions are made in the scrotum. Fong et al. (2000) describes the PESA procedure used in their facility. A local anesthetic was administered in the neck of the scrotum into the area of the spermatic cord. Another anesthetic block is applied subcutaneously anterior to the caput epididymidis. Once adequately anesthetized, the cauda epididymidis was firmly held while a 23-gauge needle was inserted into the most distended portion. A 20-ml syringe was attached to the distal end of the tubing to create negative pressure. The needle is relocated as often as necessary to obtain the amount of sperm needed to perform ICSI.

An alloplastic spermatocele procedure was developed to determine if repeated epididymal sperm collections can be executed on infertile men. Grantmyre et al. (1995), using the alloplastic spermatocele procedure in rabbits, determined that this new approach could be used to repeatedly and frequently collect epididymal sperm. This improved

spermatocele made the end small enough to allow surgical anastomosis to a single epididymal tubule similar to a vasoepididymostomy, creating a sperm reservoir that remained patent for up to 14 days. Another method of spermatocele attachment has retained patency for longer periods of time (4 weeks) (Cooper, 1990) but has not allowed recovery of equivalent numbers and/or quality of sperm as the former method. Those that have been performed in infertile men have resulted in the birth of offspring, but usually require the aid of IVF or ICSI (Muller-Tyl et al., 1990).

The final method involves collection of sperm, not from the epididymis, but from testicular tissue. Silber et al. (1995b) describes the technique for testicular sperm extraction (TESE) from infertile men. This technique is only used when there is no epididymis, when the scrotum is massively scarred or when there are no epididymal sperm. A 1-cm horizontal incision was made in the scrotal skin through the peritoneal tunica vaginalis. Next, a 0.5-cm incision was made in the tunica albuginea and a small portion of the extruding testicular tissue was removed and placed in medium. Following confirmation of sperm, the tunica albuginea and outer incision were closed. To obtain sperm from testicular tissue, the tissue is minced and the effluent is retrieved.

Methods of Sperm Analysis to Determine Fertility

There are a multitude of methods for assessing sperm and each method attempts to show a correlation to fertility. Purvis and Christiansen (1992) stated that conventional measurements of sperm quality and male fertility are inadequate and any assessment should involve several tests of sperm cell function to increase the fertility prognosis. Saacke (1983) classified sperm quality traits into two categories, either viability related or morphological traits. Viability traits are the parameters that are most affected by physiological or pathological conditions as well as the handling of the sample during

collection and processing by the technician. There are a variety of viability traits that can be measured objectively or subjectively as outlined in Table 1.1.

Table 1.1. General bases for viability measurements of sperm cells

Motility
Sperm velocity
Penetration of cervical mucus
Metabolic activity
Structural integrity of:
Cell membrane
Acrosome
Ability to pass through a density gradient (e.g., Sephadex-glass wool filter, Percoll)
Ability of sperm to bind to zona pellucida

Adapted from Saacke (1983).

Sperm Motility

Motility has long been considered a major criterion in the assessment of male fertility. The objective of assessing sperm motility is to determine the proportion of motile and progressively motile sperm in a sample (Malmgren, 1997). Saacke and White (1972) and Clarke et al. (1979) have reported that motility is a poor indicator of fertilizing potential for bovine sperm. In contrast, Gibson and Graham (1969), Linford et al. (1976) and Kjaestad et al. (1993) have reported that it is a reliable indicator of bovine fertility when used in conjunction with sperm velocity.

In a study comparing rat IVF with caput or caudal epididymal sperm, Blandau and Rumery (1964) reported that progressive motility was correlated to IVF fertility. Caput epididymal sperm move in a circular motion while cauda sperm moved in a forward pecking motion and their respective fertilization rates were 8 and 93%, respectively, for the rat. Franken (1998) stated that progressive motility is the best indicator of male fertility

for infertile couples undergoing IVF, second only to sperm-zona pellucida binding. Boyle (1996) has also reported a poor correlation between post-thaw motility and equine fertility. Even the poor correlation to fertility, Saacke and White (1972) indicated that it should be included in the sperm analysis because there are a multitude of factors that affect bovine fertility.

Percentage of progressively motile (PPM) sperm is the percentage of sperm moving in a forward direction (not a circular movement or stationary). These sperm are more likely to swim to the oocyte, whether in vitro or in vivo. A number of laboratory methods can be used to determine PPM. The traditional method of subjective microscopic estimation (Malmgren, 1997) is cheap and simple but the PPM estimates can vary greatly among and within technicians. This method can be completed with a standard microscope slide and cover slip or with gridded slides marked to aid in the ease of counting. Gridded slides include the Neubauer[®] cytometer, the Hemacytometer[®], the Makler[®] and the disposable slides such as Cell-Vu[®]. Using a specialized slide, the technician counts the number of sperm moving forward as well as sperm not moving progressively forward in a predetermined number of squares.

Another estimate of PPM can be easily evaluated using supravital sperm stains (Januskauskas and Rodriguez-Martinez, 1995). With stains and the appropriate microscope and fluorescence filters, presumably viable motile and nonmotile sperm can be easily distinguished from nonmotile sperm that are not viable. Supravital stains will be discussed in the sperm structural integrity section.

Objective methods for quantifying sperm motility are time-lapse and multiple-exposure photomicrography and frame-by-frame playback video-micrography. Photo- or video-micrography are more accurate methods of sperm motility estimation than technician

slide analysis of bovine (Revell and Wood, 1978) and equine sperm (Rousset et al., 1987) but is more labor intensive and requires more equipment (Malmgren, 1997). Acott and Hoskins (1983) used video-micrography or cinematography to determine progressive motility of bovine cauda epididymal sperm. Photomicrography and video-micrography utilize dark field microscopy and camera equipment for determining the number of forward moving sperm in a microscopic field by counting the tracks of moving sperm.

The newest method, computer-assisted sperm analysis (CASA) is possibly the most accurate and objective means of assessing PPM (Leidl et al., 1993) and other forms of motility, such as curve-linear velocity (VCL), average path velocity (VAP), straight line velocity (VSL), linearity (LIN), amplitude of lateral head displacement (ALH), beat-cross frequency (BCF) and path radius (RAD). A sample of the sperm to be analyzed is placed on a slide and subjected to video-micrography of 10 to 20 different microscope fields. A computer program (e.g., STROMBERG-MIKA[®], Cell Soft[®], Hamilton Thorn Semen Motility Analyzer[®]) then assesses those sperm recorded in the fields and places them in their appropriate categories (Mathieu et al., 1992; Slott et al., 1994; Amann et al., 2000).

Equine sperm motility and rat sperm motility have been assessed by CASA using Hamilton Thorn Semen Motility Analyzer[®] computer program (Parlevliet et al., 1992; Slott et al., 1994). Farrell et al. (1995) used the Hamilton Thorn Integrated Visual Optical System (IVOS) units with software version 10 to assess sperm parameters of the human, rabbit and bull. Another program that can be used with CASA for analysis of human sperm is the Hobson Sperm Tracker (WHO, 1999).

Sperm Velocity

Measurement of sperm velocity is a more detailed assessment of sperm motility. It is a measure of how fast the motile sperm are moving in any particular direction. Kjaestad

et al. (1993) reported the use of sperm velocity using a grading system (1 to 5), measured at 39°C with phase contrast microscopy, in conjunction with percent motility have been used as a reliable indicator of bovine fertility. Sperm velocity can also be evaluated with the aid of CASA. With CASA, sperm are observed and placed into various velocity categories such as curve-linear, average path and straight line velocity (Amann et al., 2000). Assessment of human sperm velocity using a Hamilton Thorn Semen Motility Analyzer, Liu et al. (1991) reported a positive correlation between curve-linear velocity and IVF rates for infertile couples.

Sperm Cell Structural Integrity

Sperm cell structural integrity, in combination with motility, correlates with fertility of frozen bull and stallion sperm (Linford et al., 1976; Boyle, 1996). Most of the methods for assessing the structural integrity of sperm are slow and not very repeatable (Merkies et al., 2000). Structural integrity can be measured in a variety of ways and two portions of the sperm cell are evaluated. The first is the external plasma or outer sperm membrane. If the external membrane was damaged then the DNA content would be compromised and then the sperm cell function would be compromised. The second portion of the sperm that can be evaluated is the acrosomal membrane. The acrosomal membrane encloses enzymes necessary for fertilization. If the acrosome is compromised then the sperm cells have lost their fertilizing potential. One of the more common methods used to assess sperm cellular membrane integrity is to fix a sample of the sperm solution on a slide and stain it for the differentiation of cell structure. It is best to determine the most efficient stain for a particular species before extensive use.

There are stains specific for equine sperm but general-purpose cellular stains have also been used (e.g., Wright's, Giemsa, hematoxylin-eosin) (Varner et al., 1991). Varner

et al. (1991) mentioned another group of stains, background stains, which can be used to improve sperm visualization (e.g., eosin-nigrosin, India ink). A third staining procedure involves the use of fluorescence microscopy and can be broken down into two groups, fixed sample staining and supravital staining.

Aalseth and Saacke (1986) described a combination fast green FCF-eosin B stains that was used to determine viability of bovine sperm. Rousset et al. (1987) used eosin-nigrosin staining to determine equine sperm viability. Viability of chimpanzee sperm has been determined using eosin blue-aniline blue (Young et al., 1985). Bachtell et al. (1999) used a two-stain technique to determine viability of human sperm from the vas deferens, epididymis and testis. The two stains were carboxyfluorescein diacetate (CFDA) and propidium iodide (PI). The simple dual stain procedure that uses Trypan blue to detect external membrane integrity and Giemsa to detect acrosomal membrane integrity has been used for evaluating bull, boar, buck, ram and stallion sperm (Didion et al., 1989; Chauhan et al., 1994).

Another visual assessment of sperm viability can be made with the aid of supravital stains (Januskauskas and Rodriguez-Martinez, 1995). Using these stains and the appropriate microscope and fluorescence filters, motile and nonmotile viable sperm can be easily distinguished from nonviable and nonmotile sperm. One supravital stain used for bovine sperm samples is the membrane-impermeable DNA-marker ethidium homodimer (EthD-1) (red) and the membrane-permeable cytoplasmic esterase-marker Calcein AM (green). This combination of stains can display membrane integrity without killing the sperm.

Another supravital stain that can be used for sperm viability assessment is a commercially available fluorescent double stain called Fertilight (Molecular Probes,

Eugene, OR). This stain uses two nucleic acid stains: 1) SYBR-14, which permeates intact plasma membranes allowing viable sperm to fluoresce green and 2) propidium iodide (PI), which only permeates damaged plasma membranes and causes them to fluoresce a red color (Gamer and Johnson, 1995). This supravital stain can also be used with flow cytometry to determine the number of external membrane compromised sperm in a sample (counts 10,000 sperm/sample). Gamer and Johnson (1995) validated the use of Fertilight for determining membrane integrity in the mouse, rabbit, boar, ram, bull and man. Merkies et al. (2000) reported the precision of this method for the assessment of viability for fresh equine sperm but it was less precise for frozen-thawed samples. It appears that all of the previously mentioned staining methods have a higher precision for the pattern of sperm viability when used for fresh mammalian sperm samples compared with frozen-thawed sperm samples (Gamer and Johnson, 1995; Merkies et al., 2000).

Jeyendran et al. (1984) described another method for membrane integrity analysis that does not involve staining; the hypo-osmotic swelling (HOS) assay can be used to measure sperm viability. This is a simple test based on the permeability of the intact sperm cell membrane allowing the sperm tail to swell under hypo-osmotic conditions. This test also gives information on the integrity of the sperm tail membrane by showing a curl in the tail if viable (WHO, 1999). As a selection measure, the HOS assay identified significantly more viable epididymal and testicular sperm than did motility in both fresh and frozen-thawed samples.

The second area of structural integrity is the acrosomal region. Integrity of the acrosomal membrane is important because of the role it plays in fertilization. If the acrosomal membrane is interrupted then sperm function is compromised and fertilization does not take place. This region of the acrosome can be assessed by several methods,

including wet or fixed samples that are unstained or stained by the use of differential interference contrast microscopy (DIC). Cross and Overstreet (1987) reported that with the use of lectin labels combined with fluorescein isothiocyanate (FITC) it is possible to differentiate between sperm that are acrosome reacted compared with sperm that are acrosome intact.

The lectin label wheat germ agglutinin (WGA) method was correlated with male fertility in humans (Franken, 1998). Lectin proteins used to label membrane surface saccharides are referred to as “agglutins”. These labels/stains attach to the carbohydrate moieties on the sperm cell surface, which change as the acrosome reaction occurs (Magargee et al., 1988; Bawa et al., 1993).

Spermac[®], as a stain for determining intact acrosomes has been reported to be successful for evaluating bovine, equine and ovine sperm cells (Parlevliet et al., 1992; Kjaestad et al., 1993; Byrne et al., 2000). Another simple stain, fast green FCF/rose bengal was developed to assess acrosomal integrity of cat sperm (Pope et al., 1991). Kholkute et al. (1995) reported the use of chlortetracycline (CTC) to measure capacitation changes that had occurred in mice sperm and Morris et al. (2000) reported its successful use in stallions. The CTC staining method is used for sperm that are fixed and reveals three or four staining patterns for analysis of capacitation. The first pattern is uniform fluorescence that is associated with uncapacitated, acrosome intact sperm. The second pattern has a fluorescent-free zone in the post-acrosomal region and is associated with capacitated, acrosome-intact cells. The third pattern has dull or no fluorescence of the sperm head and represents capacitated and acrosome reacted cells.

Fleming et al. (1976) and Chandler et al. (1978) reported the percentage of sperm with intact acrosomes after 2 or 4 hours of incubation at 37°C was highly correlated to

fertility in bulls. They used DIC microscopy to assess the apical ridge, which is present in sperm with an intact acrosome. The validity of DIC to measure acrosomal integrity was confirmed by ultrastructural examination (Aalseth and Saacke, 1985). Differential interference contrast microscopy can be used with a wet sperm preparation or a fixed preparation, and therefore, has an advantage to the sperm stains that require a fixed sample. Fixed samples can have artifacts created by the fixing process, which can damage some cells thus altering the results. Steinholt et al. (1991) compared brightfield and DIC microscopy for evaluation of intact acrosomes of bull sperm and found that both methods were equally accurate.

Oehninger et al. (2000) assessed membrane integrity of post-thaw sperm with annexin V. Annexin V-binding measures the translocation of phosphatidylserine (PS) from the inner to the outer layer of the plasma membrane to aid the detection of the deterioration of membrane functions (Glander and Schaller, 1999). The study assessed membrane integrity and correlated those results with motility changes. It was determined that the degree of annexin V binding measured in the sperm fractions with high motility may be used to predict the ability of the sperm to survive cryopreservation.

Ability of Sperm to Bind to Zona Pellucida

A valuable assessment of the fertilizing ability of bull sperm can be obtained by a combined analysis of sperm linear-motility patterns, swim-up separated motility and homologous sperm-zona pellucida binding (Zhang et al., 1992). Boyle (1996) reported the use of zona or oocyte binding assays to assess equine sperm fertility. Although it is an accurate method for assessing in vivo fertility it is too complex for on-farm use. Franken (1998) also reported the use of a sperm-zona pellucida binding assay as a means of determining fertility or etiology of infertility in couples. Many independent laboratories

have confirmed that these tests are good indicators of fertility in men. Two types of sperm binding tests were reported by Franken (1998), the hemizona assay (HZA) and the competitive intact zona pellucida-binding test. The HZA test was developed to make the most efficient use of scarce human zonae because it has a built-in control ($\frac{1}{2}$ zona pellucida = test sperm; $\frac{1}{2}$ zona pellucida = control sperm).

Studies have been reported on the functional integrity of the sperm plasma membrane of frozen-thawed sperm. Of the sperm membrane features examined, the use of tight sperm-zona pellucida binding by HZA has been positively correlated with fertility (Oehninger et al., 2000). Cryopreserved donor sperm in the HZA assay exhibited a mild decrease of binding capacity. Amann et al. (1999) evaluated a unique method for the determination of the percentage of sperm that might bind to the zona pellucida. Instead of using HZA they used a 60-minute incubation period with extracts of chicken perivitelline membrane coated in microwell assay plates. The human zona pellucida and the inner laminar perivitelline membrane of the chicken share analogous structures that should allow sperm binding with similar results. It was concluded that too much inter- and intra-plate variation occurred to be accurate and further studies are needed to determine if the binding ability is correlated to fertilization in vitro.

Cryopreservation of sperm can reduce motility and may also decrease the energy production and reserve system of sperm (Oehninger et al., 2000). Such damage makes assessing energy status of cryopreserved sperm less informative. The more useful assessments of cryodamage relate to time and functional and structural integrity of the sperm plasma membrane. These tests include eosin-Y (a membrane stain), HOS and fluorophores, such as PI (Jeyendran et al., 1984; Lorneo and Giambersio, 1991; Alvarez et al., 1993; Garner and Johnson, 1998; WHO, 1999). Esteves et al. (1996) stated that HOS

could accurately predict the viability of fresh human sperm but not that of cryopreserved samples. Lin et al. (1998) determined that neither HOS nor eosin-Y could effectively predict sperm motility after the freeze-thaw process.

Magargee et al. (2000) reported the use of an in vitro assay for sperm-ovum binding to determine the binding and fertilizing capacity of sperm from different mouse strains. This test relies upon an ovum-membrane substrate coated on a plate. Sperm are incubated with this plate and the number of sperm bound/mm² make up the results. This in vitro assay could readily identify sperm that had low sperm-oocyte binding capabilities the results were also correlated to IVF rates. Also, Rojas et al. (1992) used an assay involving the penetration of zona-free hamster oocytes to test the fertility of epididymal sperm aspirated from men with congenital absence of the vas deferens (CAVD). The penetration values for the epididymal sperm were lower than those of ejaculated sperm but were significantly correlated with IVF results. It was determined that this assay was an efficient method for assessing fertility of aspirated epididymal sperm.

Various Methods of Sperm Assessment for Different Species

The following sections will describe some of the sperm assessment methods used for selected species in particular situations. Morrell et al. (1997) used conventional tests for sperm quality of marmoset monkeys, including concentration (Neubauer[®] cytometer) (Moss et al., 1979); subjective estimation of motility, live:dead ratio (Blom, 1950), and morphology using Testsimplets[®] (staining procedure) (Schoenfeld, 1981). Human IVF clinics currently use the Kruger's strict criteria to determine morphologic normalcy for human ejaculated and epididymal sperm (Kruger et al., 1986; WHO, 1999) and assessment of sperm morphology for normalcy has been shown to be correlated to in vitro fertilization.

Thundathil et al. (2001) reported an inverse relation between IVF rates and percentage of proximal cytoplasmic droplet retention in the bull. An increased retention of cytoplasmic droplets was considered standard for epididymal sperm in comparison with ejaculated sperm from the domestic bull (Gupta et al., 1996) and the African buffalo (Bartels et al., 1998). Feline sperm was reported to be highly abnormal (teratospermic) with epididymal sperm showing a greater proportion of distal or cytoplasmic droplets than ejaculated sperm (Axner et al., 1998).

Brandon et al. (1999) reported on the correlation of several seminal plasma proteins and fertility in stallions. A positive correlation was found between fertility and a seminal plasma protein called SP-1 (72 kDa, pI 5.6). For the seminal plasma proteins, SP-2, SP-3 and SP-4 (75 kDa, pI 6.0; 18 kDa, pI 4.3; 16 kDa, pI 6.5) a negative correlation was made with fertility. Brandon et al. (1999) stated that identification of these proteins in stallion seminal plasma could aid in the study of infertility in the horse.

Patrizio et al. (1994) described a unique measurement to determine the fertility of epididymal sperm collected from men with congenital absence of the vas deferens (CAVD) to be used for in vitro procedures. The length of the epididymis was measured and a positive correlation was made between length and fertility. Those patients who had longer epididymides were more likely to achieve pregnancy and produce an offspring through assisted reproductive technologies. They considered the reasons for increased fertilization rates to be that a longer epididymis can allow 1) the arrival of more frequent waves of fresh sperm, 2) less obstructive damage and 3) a back flow of biochemical factors produced in distal segments that could enhance fertilization capacity of proximal epididymal sperm. This method is not a useful test for males with normal testicular function because it is related to the damage created to sperm cells because of an extended

retention period at the end of the epididymis nearest the missing portion of the reproductive tract.

Cryopreservation and Cool Storage of Sperm

Woelders (1997) has emphasized that the process of cooling, freezing and thawing subjects sperm to a number of changes in their immediate environment. The challenge during freezing is not the ability to survive very low temperatures but to survive an intermediate zone of temperatures (-10° to -50°C) that the cells go through twice during a freeze-thaw procedure (Saacke, 1983). The nature of cellular damage by cryopreservation involves two primary factors. The first is exposure to hypertonic conditions that occur because of the removal of water by ice, creating an area of high salt concentrations or the “solution effect” (see reviews by Holt, 2000a, 2000b; Vishwanath and Shannon, 2000). The second is the formation of intracellular ice that physically pierces cellular membranes. Check et al. (1994) reported that decreasing the time of sperm exposure to ice forming temperatures increases the cryosurvivability. Woelder (1997) and Holt (2000a) reviewed the changes that occur during cryopreservation beginning with the cooling phase. The sperm membrane lipids undergo phase transition and the proteins are altered, affecting the structural integrity and ion metabolism in the sperm.

White (1993) reported that the high unsaturated:saturated fatty acid content in combination with low cholesterol content makes sperm more susceptible to cryoinjury. The high unsaturated fatty acid content increases the sperms susceptibility to hydroxynonenal (HNE), a fatty acid product of acid peroxidation. HNE causes an irreversible loss of motility, inhibition of fructolysis and respiration, leakage of intracellular enzymes and structural damage to the plasma membrane in vitro, particularly the acrosomal region of ovine sperm (White, 1993).

The next portion of the cryopreservation process, freezing, may lead to extracellular ice nucleation and osmotic changes that produce an efflux of water from sperm. This will affect a loss of stability of the lipid bilayer by removing membrane proteins, called “membrane leakiness” (Woelder, 1997; Holt, 2000a, 2000b). Janny and Menezo (1994) proposed a link between cryoinjury and the early events of fertilization and embryo development in humans. Other damage from this portion of cryopreservation include an alteration of the sperm membrane components because of delayed reactions, lipid peroxidation, or the phase transition may make the membrane more prone to early fracture at stress points (Alvarez and Storey, 1992; Alvarez et al., 1993; Drobnis et al., 1993; Holt, 2000a).

Other consequences of cryopreservation on sperm include denaturation of proteins, structural deformation of cellular organelles and eutectic crystallization of intracellular solutes, leading to cellular death (Saacke, 1983; Alvarez and Storey, 1992; Alvarez et al., 1993; Drobnis et al., 1993). Saacke (1983) proposed that these injuries are dependent upon the length of time that sperm are exposed to the dangerous temperature zone and others have indicated that increasing the freeze-thaw rate would minimize exposure to damage for bovine (Robbins et al., 1976; Almquist et al., 1982), equine (Klug et al., 1992) and ovine sperm (Byrne et al., 2000).

Canine epididymal sperm has been reported to be frozen in test yolk buffer plus 3% glycerol (Stilley et al., 1999). The freezing protocol cooled samples to 4°C and then placed them in LN₂ vapors for 10 minutes before plunging into LN₂. It was found that canine epididymal sperm can survive a freeze-thaw procedure after being stored within the cauda epididymidis for up to 48 hours at 4°C. Yu and Leibo (2002) preserved canine

epididymal sperm in the testicle for up to 8 days postmortem at 4°C. On day 8, the sperm exhibited 50% progressive motility and 80% membrane integrity.

Several groups have reported successfully cryopreserving porcine epididymal sperm that was collected immediately postmortem (Rath and Niemann, 1997; Ikeda et al., 2002) or stored in the testicle at 4°C for up to 3 days and cryopreserved (Kikuchi et al., 1998). Blandau and Rumery (1964) have stated that epididymal sperm from the ram, boar and bull were more resistant to cold shock than ejaculated sperm. Caprine sperm has been stored at temperatures ranging from 2 to 15°C, with the best success at 4 to 5°C (Leboeuf et al., 2000). It has been reported that no systematic investigations have been completed on diluent compositions required for preservation of caprine sperm. Cryopreservation of caprine ejaculated sperm involves washing to remove sperm from the seminal plasma. Leboeuf et al. (2000) described a method for freezing caprine sperm by first washing then resuspending that sample with half the final volume using the nonglycerol portion of the cryoprotective medium. The resuspended caprine sperm sample is then cooled from 30° to 4°C over 1 hour and further diluted with the portion containing 14% glycerol. When completely resuspended the samples are frozen by suspending straws 4 to 5 cm above the LN₂ for 4 to 5 minutes and then plunged into LN₂. There was no clear preference established for cryopreservation of caprine sperm with any extender evaluated. The most popular extenders are reconstituted cow skim milk-glucose extenders or tris-glucose-citric acid-yolk extenders. It was established that ethylene glycol and propylene glycol gave less protection to caprine sperm than did glycerol and that the optimum concentration was between 4 to 7% in diluted sperm.

Keskintepe et al. (1998) compared caprine sperm cryopreservation with either a skim-milk based extender compared with an egg-yolk based extender. Successful IVF

occurred with sperm preserved in the skim-milk extender. The egg-yolk extender enabled good sperm motility post-thaw but IVF with the frozen-thawed goat sperm was compromised.

Epididymal sperm collected at necropsy from a dairy goat male were successfully cryopreserved (Blash et al., 2000). The sperm were cryopreserved in a medium with 20% egg yolk and 7% glycerol. The frozen-thawed samples exhibited a 10% decrease in percent viable sperm from the prefreeze to the post-thaw evaluation.

Equine sperm cryopreservation has had only limited success because of high variability among individual stallion sperm parameters. Unlike bulls, stallions have not been selectively mated over time in domestic situations for reproductive purposes. This has the unfortunate effect of creating an animal model that is very difficult to cryopreserve by any one method. Some of the more common extenders used for stallion sperm include a simple skim milk extender by Kenney et al. (1975) that has been altered by the addition of egg yolk and glycerol (Parlevliet et al., 1992). Another extender just uses egg yolk with glycerol as the cryoprotectant (Parlevliet et al., 1992). Equine sperm collected from the cauda epididymidis after 24 hours of storage at 5°C are capable of surviving cryopreservation as assessed by post-thaw motility (0% to 27%) (Bruemmer et al., 2002). Tiplady et al. (2002) collected equine epididymal sperm, cooled the sperm to 4°C, then cryopreserved and assessed the sperm at post-thaw for motility and membrane integrity. These sperm samples exhibited a decrease in motility but not in viability from prefreeze to post-thaw.

Klug et al. (1992) reported that the cooling rate to 4°C for stallion semen is very important and should be slow while the freezing rate, as well as the thawing rate should be much faster to retain viability. Vidament et al. (2000) determined that the addition of

glycerol while cooling equine sperm from 22°C to 4°C at a moderate rate (1 hour) improved post-thaw motility and per cycle fertility. Morris et al. (2000) have reported that epididymal sperm collected immediately after castration was cryopreserved in Tris-egg-yolk-glycerol diluent. These sperm were frozen in LN₂ vapors and plunged into LN₂. The fresh epididymal sperm exhibited 75% viability while frozen-thawed epididymal sperm had 30% viability.

Arav et al. (1994) has emphasized that the thawing procedure was critically important to the survival of equine ejaculated sperm. They also reported a better survival rate for sperm cryopreserved with thermal hysteresis proteins and thawed using a 75°C water bath for 10 seconds followed by 30 seconds in a 38°C water bath when compared with a 38°C water bath for 45 seconds or a 23°C water bath for 80 seconds.

Vilakati and Summers (1996) incubated frozen-thawed bovine ejaculated semen with a molecular component that is greater than 10 kDa isolated from the cauda epididymidis. Incubation with this protein improved the sperm viability over time post-thaw but it had no effect on sperm motility. It was suggested that this molecular component is responsible for sperm cell membrane maintenance in the cauda epididymidis.

Kilian et al. (2000) cryopreserved epididymal sperm collected from African wild ruminant species, including the Black wildebeest, blesbok, Roan antelope, gemsbok, nyala, eland and African buffalo. The sperm were cryopreserved in either a Tris-buffered diluent (Biladyl; Minitube, Germany) or in Hams F-10 (with sodium bicarbonate but without glutamine). The cryopreservation protocol included 6 hours equilibration at 4°C, 15 minutes in LN₂ vapor followed by plunging into LN₂. Loss of motility was less for samples preserved in Biladyl medium (a variation of egg-yolk citrate medium).

Loskutoff et al. (1996) examined the toxicity and freezing resistance of epididymal sperm of three species of African antelopes (blesbok, impala and springbok) exposed to glycerol, ethylene glycol (EG), propylene glycol (PG) and dimethyl sulfoxide (DMSO). In this study, glycerol was found to be least toxic when incubated with sperm at 30 to 32°C. Also, both glycerol and EG were found equally effective cryoprotectants for blesbok and impala sperm. The springbok sperm survived cryopreservation best with the DMSO cryoprotective agent.

Schmid et al. (1997) compared four different extenders for the kudu. One extender was an egg yolk, Tris-TES with 7% glycerol, two were skim-milk based with either salts or sugars and 3% glycerol and the last was lactose EDTA with 20% egg yolk and 5% glycerol. It was determined that the egg yolk, Tris-TES with 7% glycerol medium best preserved motility and viability after 3 hours of incubation of frozen-thawed kudu epididymal sperm. A comparison of Biladyl Minitube (egg-yolk, tris-TES based medium) with either glycerol or ethylene glycol for impala epididymal sperm was reported to have higher motility and viability than those sperm maintained in Biladyl medium with glycerol (Rush et al., 1997). Epididymal sperm collected 27 hours postmortem from gaur bulls was cryopreserved and assessed post-thaw for survival (Hopkins et al., 1988). Initial post-thaw motility was high but after 2 hours of incubation the sperm exhibited 0% motility.

Lubbe et al. (2000) describe cryopreservation of epididymal sperm collected from the horse, zebra and rhinoceros comparing two different cryodiluents. The sperm were frozen with either a skim-milk based glycerol equine diluent (PathVet, RSA) or Biladyl (Minitube, Germany) an egg-yolk based diluent. Samples were collected and equilibrated for 6 hours with a final glycerol concentration of 3 to 3.5%. After equilibration the straws were placed in LN₂ vapors for 15 minutes and then plunged for the final freeze. There was

no difference detected between the post-thaw results of the two media for equine, zebra or rhinoceros epididymal sperm cryopreservation.

In another study, Anel et al. (1999) recovered epididymal sperm from the Cantabric brown bear no more than 70 minutes postmortem. The sperm were cryopreserved and later thawed for analysis. Prefreeze analysis included the percent of epididymal sperm that retained a cytoplasmic droplet (82%), percent progressive motility (30%) and the percent sperm with an intact acrosome (62%). Analysis of the thawed samples resulted in 15% progressive motility and 29% with intact acrosomes. These sperm samples exhibited a 50% decrease from prefreeze to post-thaw for both percent progressive motility and the percent sperm with an intact acrosome.

In the African elephant various cryoprotectants have been investigated as to effects on epididymal sperm by the reproductive physiology group at the Omaha Henry Doorly Zoo (Hoffman, personal communication). The sperm were exposed to glycerol, EG, PG and DMSO to evaluate toxicity and cryoprotective abilities. Elephant epididymal sperm exhibited sensitivity to the EG and PG cryoprotectants but no toxic effects were noted with glycerol or DMSO. Cryosurvivability was greater for samples frozen with glycerol or DMSO while samples with EG or PG were found to have <10% motility post-thaw.

Sperm parameters and location of aspiration in humans were examined by Sharma et al. (1997) for correlation with epididymal sperm and ability to survive cryopreservation and achieve fertilization and pregnancy by ICSI. In this study, none of the sperm parameters or aspiration location could be associated with ability of epididymal sperm to survive cryopreservation. Bachtell et al. (1999) compared the viability of human fresh and frozen sperm retrieved from the vas deferens, epididymis and testis. Fresh sperm from the vas deferens were more motile but testicular sperm exhibited a high viability (91 and 96%,

respectively). Sperm from testis, epididymis and vas deferens had similar parameters when assessed by vital stain but not for motility after freezing.

Recently, Patrizio (2000) has described a protocol for human epididymal sperm cryopreservation as washing sperm, concentrate to a volume of 0.3 to 0.6 ml and diluted 1:1 with freezing medium (TEST-yolk buffer with glycerol). Freezing medium was slowly added over a period of at least 10 minutes, while the solution was continuously mixed. Vials were suspended 2.54 cm above the meniscus of the LN₂ overnight and plunged into LN₂ the next day.

In addition, Hsieh et al. (2000) described a unique method of storage for human epididymal sperm for cryopreservation that involved placing the sperm cells within an empty human or mouse zona pellucida. They developed this method when seeking a more efficient method to preserve the epididymal sperm collected from men who suffer severe oligoasthenozoospermia and who have a total sperm number too low to freeze with conventional methods. Cohen et al. (1997) have reported a method for single sperm freezing that was an improvement over placing several sperm within a zona pellucida. Both groups used an empty zona pellucida as a vehicle for storing and freezing from 1 to 20 sperm. Cohen et al. (1997) suggested that 1 to 3 sperm frozen within a zona was optimal, however, Hsieh et al. (2000) reported similar recovery rates when preserving 15 to 20 sperm/zona pellucida.

Use of Epididymal Sperm for AI

Blandau and Rumery (1964) compared fertilization after AI of caput versus cauda epididymal sperm from the rat. Caput sperm had 8% fertilization while cauda sperm had 93% fertilization. Rat offspring were produced from intrauterine insemination (IUI) with cryopreserved epididymal sperm (Nakatsukasa et al., 2001).

A comparison of litter size produced was made between proximal and distal cauda epididymal sperm used for AI in the guinea pig (Young, 1931). A fertilization rate of 68% and 33% and an average litter size of 2.6 and 1.8 were produced for distal and proximal cauda epididymal sperm, respectively.

Paufler and Foote (1968) produced litters of rabbits after AI with epididymal sperm from both the caput and cauda epididymidis. Fetal development was reported in the rabbit after IUI with corpus and cauda epididymal sperm but no offspring was reported (Orgebin-Crist, 1969).

Marks et al. (1994) reported the production of a canine pup from AI with frozen-thawed epididymal sperm. The entire sperm sample collected and cryopreserved was used to inseminate one bitch. The prefreeze sample had a 60% progressive motility while the post-thaw sample exhibited only a 5% progressive motility rate.

Holtz and Smidt (1976) reported fertilization using AI technology when comparing the fertilizing capacity of pig epididymal sperm extracted from different regions of the epididymis. Porcine caput epididymal sperm had an 8% fertilization rate with rates improving as the samples progressively neared the cauda epididymidis, with 83% fertilization. In this study, every segment of the porcine epididymis produced live fetuses from AI.

Blash et al. (2000) reported the use of frozen caprine epididymal sperm for the AI procedure. Overall, 20 females were inseminated with the frozen sperm, and one doe became pregnant. This one pregnancy resulted in the production of one healthy kid.

Barker and Gandier (1957) reported the birth of a foal produced by AI with frozen-thawed equine epididymal sperm. The epididymal sperm was frozen to -79°C and later used for AI in two doses to seven mares exhibiting estrus. More recently, pregnancies

were reported using frozen-thawed equine epididymal sperm during hysteroscopic insemination (Morris et al., 2001).

Bovine epididymal sperm was harvested immediately postmortem and cryopreserved and stored at -79°C. At the time of publication this sperm had been thawed and used for AI to achieve pregnancy (Barker, 1954). Igboeli and Foote (1968) reported the use of cauda epididymal sperm stored at 5°C for 60 hours postmortem was for AI, which resulted in a 69% nonreturn rate in cattle. The fertility of bovine epididymal sperm was compared with ejaculated sperm using AI (Amann and Griel, 1973). Fertilization was achieved by AI with both sperm sample types, with epididymal sperm resulting in an 84% and ejaculated sperm showing a 94% fertilization rate. Foote (2000) reported the use of bovine epididymal sperm recovered from four slaughtered bulls and then stored at 5°C for ~60 hours. It was processed for AI and exhibited a nonreturn rate of 69%.

The birth of an eland following AI with frozen-thawed epididymal sperm was reported (Bartels et al., 2001). Sperm were maintained at 4°C for 5-hours postmortem before processing at a laboratory for cryopreservation. Average post-thaw motility was 55%. Morrell et al. (1997) stored epididymal sperm collected from marmoset monkeys in test yolk medium at 4°C until required for AI up to 48 hours after collection. Live births were achieved by AI with this cooled epididymal sperm. Pregnancies have been achieved in the human with intracervical inseminations (ICI) of epididymal sperm collected from an artificial spermatocele (Cooper, 1990).

Use of Epididymal Sperm for IVF

Mouse IVF research involves the use of epididymal sperm because of the ease of collection compared with collecting ejaculated sperm. Miyamoto and Chang (1972) reported development of mouse embryos by IVF with epididymal sperm. They achieved

64% fertilization, 10% blastocyst development and 13% of transferred embryos developed into normal fetuses. Tada et al. (1994) reported 54% in vivo fetal development after embryo transfer of embryos produced by IVF with frozen-thawed mouse epididymal sperm and vitrified oocytes. Kholkute et al. (1995) increased fertilization rates with the addition of progesterone to the IVF culture system in mice. Progesterone was reported to have a positive effect on the acrosome reaction in different species (e.g., hamsters, mice, pigs, horses and humans) and was found in the follicular fluid of the human.

Kuzan et al. (1990) reported that platelet-activating factor (PAF) may play an important role in mouse oocyte fertilization. When exogenous PAF was added to culture there was an increased fertilization rate while the addition of an antagonist led to the supernumerary attachment of sperm but decreased the fertilization rate. In another mouse IVF study, Songsasen et al. (1997) used cryopreserved mouse epididymal sperm to produce live mouse pups. With the use a raffinose-glycerol solution containing egg yolk for cryopreservation a 26% blastocyst development rate and a 34% live pup production per embryo transferred were obtained. These results were similar to the results obtained by IVF with fresh mouse sperm samples (Songsasen et al., 1997). Toyoda and Chang (1974) reported the use of IVF to produce embryos and live offspring with epididymal sperm from the rat. Fertilization rate was reported to be 88.7% and the development to live offspring was 21%.

Niwa et al. (1985) assessed fertilized cat 2n 1-cell pronuclear status of ova at 4 hours post-insemination after IVF with epididymal sperm to determine the length of time to acrosomal reaction. It was determined that there was pronuclear formation at 4 hours and this was indicative of the very quick capacitation of feline sperm. More recently, Pushett et al. (2000) reported successful IVF using feline frozen-thawed epididymal sperm

extracted post-castration incubated with in vitro matured ova extracted from ovaries after spaying domestic females. A cleavage rate of 30.8% and developmental rate to the blastocyst stage of 40% was reported in this study.

Nagai et al. (1988) reported the use of frozen-thawed porcine epididymal sperm that was harvested <3 hours postmortem at ambient room temperature compared with frozen-thawed ejaculated sperm for IVF. The epididymal sperm penetrated ova 0% to 40% of the time compared with ejaculated sperm penetrating 0%. Porcine epididymal sperm produced embryos at the 2-cell stage at a rate of 51%. One piglet was produced from this study using frozen-thawed epididymal sperm. Rath and Niemann (1997) and Ikeda et al. (2002) reported the use of frozen-thawed porcine epididymal sperm for IVF. Porcine epididymal sperm was collected immediately postmortem and cryopreserved. The cryopreserved epididymal sperm was compared with the cryopreserved ejaculated sperm. The frozen-thawed porcine epididymal sperm had a higher rate of fertilization (60% compared with 16%) and a higher rate of development to the 2-cell stage (21% compared with 5%) than frozen-thawed ejaculated sperm. Ikeda et al. (2002) reported higher rates of sperm penetration for IVF if frozen-thawed porcine epididymal sperm (61 to 80%) were pre-incubated prior to culture with oocytes for up to 60 minutes. Fertilization rates with pre-incubated epididymal sperm were found to be dependent upon the percent of intact acrosomal membranes instead of the percent progressive motility. Kikuchi et al. (1998) reported a greater percent loss of intact acrosomes in porcine epididymal sperm for the longer pre-incubation periods. The epididymal sperm was stored in the epididymis for 24 hours at 4°C to cryopreserving. This sperm was then used for IVF after thawing and achieved fertilization.

Fresh and frozen-thawed caprine epididymal sperm collected immediately postmortem and cryopreserved in a glycerol egg-yolk based extender was used for in vitro production of embryos (Song and Iritani, 1988; Keskinetepe et al., 1998; Blash et al., 2000). Blash et al. (2000) used frozen-thawed sperm for IVF and had a fertilization rate of 40% and a blastocyst development rate of 6%. When these values are compared with the success of IVF with cryopreserved ejaculated semen there was no difference between the values at the different embryo stages.

Brackett et al. (1982) were first to report the birth of a live calf produced by IVF with ejaculated bull sperm. Ball et al. (1983) reported a fertilization rate with IVF of 40 to 43% and up to 90% with bovine epididymal sperm. Graff et al. (1996) have reported pregnancies after IVF with bovine epididymal sperm collected via epididymal aspiration of a live bull. Two ongoing pregnancies were reported at the time of publication. This shows that embryos can be developed by IVF with epididymal sperm from domestic cattle.

Winger et al. (1997) used blesbok frozen-thawed epididymal sperm for IVF and achieved fertilization assessed by oocyte fixation. The in vitro production of African buffalo embryos was accomplished with extracted ova and epididymal sperm (Shaw et al., 1995). The epididymal sperm was collected from culled males and transported at 4°C for up to 24 hours. The sperm was extracted and processed for immediate use with the harvested ova resulting in 18 to 50% cleavage rates and 33 to 65% embryo development rates to the morula stage. None of the embryos produced during this experiment continued development to the blastocyst stage.

Epididymal sperm was collected from a free ranging adult male lion by cauda epididymectomy, cryopreserved and used for IVF of in vitro matured lioness ova (Bartels et al., 2000). Epididymides were transported to the laboratory at 4°C where they were

punctured to retrieve epididymal sperm. Sperm were frozen in Triladyl medium (Minitube, Germany). Post-thaw motility ranged from 55 to 65% and the percentage of fertilized ova was 12.7% for 30 hours of in vitro maturation and 11.5% for 36 hours of maturation.

Pryor et al. (1984) reported the first human pregnancy achieved using IVF techniques with sperm aspirated from the vas deferens. This pregnancy resulted in miscarriage. Temple-Smith et al. (1985) reported the first pregnancy from IVF with MESA sperm from a patient with a failed vasectomy reversal. Silber et al. (1987) reported the first two pregnancies from IVF achieved with MESA sperm from two patients with bilateral congenital absence of the vas deferens (BCAVD). The first reported birth after fertilization with epididymal sperm from a male patient with obstructive azoospermia using IVF was 1990 (Silber et al., 1990). Mathieu et al. (1992) stated that IVF with epididymal sperm collected from men with BCAVD could be improved by treatment of sperm with density gradient separation. Higher quality sperm are concentrated and made available to the ova during IVF with density gradient separation.

Use of Epididymal Sperm for ICSI

Intracytoplasmic sperm injection is currently used for three types of sperm: 1) sperm collected from ejaculated semen, 2) epididymal sperm and 3) testicular sperm (Tesarik et al., 1995; Van Steirteghem et al., 1998). Roknabadi et al. (1994) reported the birth of seven mouse pups after ICSI with epididymal sperm. Eighteen live rat pups were produced following ICSI with frozen-thawed transgenic epididymal sperm (Hirabayashi et al., 2002). Transgenic epididymal sperm was cryopreserved and stored at -20°C and used with a modified ICSI technique known as the Piezo method. The successful production of offspring was thought to be due to the decreased damage of the ooplasm caused by a

standard ICSI pipette. Hosoi and Iritani (1993) reported the live birth of two normal young after the transfer of 72 2- to 4-cell stage rabbit embryos produced by ICSI with fresh epididymal sperm.

Pushett et al. (2000) reported fertilization results using ICSI in the domestic cat that were comparable with those reported by Nagy et al. (1995) for humans. The former study reported better results using ICSI compared with IVF (54.3% fertilization rate and 57% blastocyst development for ICSI) to achieve fertilization and blastocyst development with cryopreserved epididymal sperm and IVM-produced feline ova. Therefore, the use of domestic cat epididymal sperm to achieve fertilization by IVF is possible but better results would likely be obtained with ICSI. Pope et al. (1998) compared fertilization rates and blastocyst development rates following IVF (68 and 53%, respectively) or ICSI (58 and 43%, respectively) using feline ejaculated sperm and found that they were comparable with those rates reported for epididymal sperm. Pope et al. (1998) also reported the production of three kittens following the transfer of ICSI-derived embryos. More recently, Bogliolo et al. (2001) reported an 82% fertilization rate and 7% blastocyst rate after ICSI with cryopreserved feline epididymal sperm.

Goto et al. (1990) first reported the birth of a live calf after transferring bovine embryos developed from immobilized, killed epididymal sperm. A later study reported the live births of two calves after transfer of bovine embryos created by ICSI with bovine epididymal sperm (Goto, 1993). The latter study reported blastocyst development rates of 7.3% for bovine embryos produced using the ICSI technique with frozen-thawed epididymal sperm. Successful use of ICSI for bovine calf production has been very difficult to achieve. Recently, Hamano et al. (1999) used ejaculated sperm heads that had been sorted by flow cytometry for bovine ICSI. When used for ICSI, the sorted sperm

heads generated a 46.6% cleavage rate and a 6.9% blastocyst rate. On day 7 through 8, 48 blastocysts were individually transferred to recipient cows resulting in a 20.8% pregnancy rate and a 20.8% normal live offspring production rate for a total of 10 calves.

Liow et al. (2002) reported the birth of two cynomologous monkeys after ICSI with frozen-thawed epididymal sperm. Intracytoplasmic sperm injection was first attempted with human oocytes and ejaculated sperm in 1988 (Lanzendorf et al., 1988). The first live offspring resulting from ICSI with human ejaculated sperm was reported by Palermo et al. (1992). The birth of four infants was reported by Mathieu et al. (1992) resulting from ICSI with human epididymal sperm.

The use of round spermatids with ICSI has been attempted and the first human baby was reported by Tesarik et al. (1995). Nagy et al. (1998) reported on the influence of some sperm parameters and success when using the ICSI technique. Only two rare conditions had a strong negative effect on ICSI outcome when dealing with ejaculated sperm. When sperm that were immotile (presumably dead) or had rounded heads (globozoospermia) were injected in 82 IVF cases the fertilization rates decreased to 19% resulting in only one set of twins being delivered at term. Silber et al. (1995a) and Nagy et al. (1998) reported that injection of motile testicular sperm increased fertilization rates of ICSI. Use of ICSI will bypass most of the sperm/female tract/ova interactions by placing the sperm inside of the ova. This aids the production of embryos with sperm samples that contain immobile sperm produced by cryopreservation procedure.

Intracytoplasmic sperm injection, in contrast to standard insemination, provides high fertilization and embryo developmental rates when using epididymal and testicular sperm from infertile men who suffer from either CBAVD or irreparable obstructive azoospermia (Silber et al., 1994, 1995b; Nagy et al., 1995; Schlegel et al., 1995; Madgar et

al., 1996). Silber et al. (1995b) reported that when MESA failed and TESE was required to retrieve sperm, ICSI still produced embryos, blastocysts and even offspring. Success makes this an alternative for couples with severe male infertility. Clinical laboratory results produced success with sperm from men with sperm deficiencies created by nonobstructive azoospermia etiologies, such as incomplete Sertoli-cell only syndrome, maturational arrest, hypospermatogenesis and even a block in meiosis.

Garrels et al. (1998), Van Steirteghem et al. (1998), Tournaye et al. (1999), Janzen et al. (2000) and Cayan et al. (2001) reported a comparison of ICSI outcomes with fresh and frozen epididymal sperm from humans. It was determined that there was no difference in success rates for fertilization, transfer or production of offspring when comparing fresh with frozen epididymal sperm from infertile couples with obstructive azoospermia. Patrizio (2000) reported no difference in fertilization and pregnancy rates between fresh and frozen-thawed ICSI cycles using human epididymal sperm collected by PESA.

A healthy baby boy was born following embryo production by ICSI with epididymal sperm from a male partner that was a carrier for cystic fibrosis (CF) (Liu et al., 1994). These embryos were subjected to preimplantation genetic diagnosis prior to transfer to decrease the chance of transmitting CF to the offspring. Patrizio et al. (1995) reported the live birth of a healthy baby girl after ICSI with epididymal sperm that had been cryopreserved after spending 24 hours in microdrops for an IVF procedure. Upon thawing for the second cycle enough sperm were motile and used to create embryos with the ICSI procedure.

CHAPTER 2

PRESERVATION OF RAT EPIDIDYMAL SPERM STORED IN THE EPIDIDYIMIDES OF TESTES AT 4°C FOR 24, 48, 72 AND 96 HOURS

Introduction

Recently, epididymal sperm has become an increasingly researched area in the field of animal reproduction. This would be especially important for research concerning the preservation of endangered species. Epididymal sperm can be used as a valuable genetic tool to maintain variety in captive populations. Even more valuable, would be the ability to retrieve epididymal sperm for either germplasm banking or the production of offspring from a recently deceased or culled male of almost any mammalian species. When working with postmortem specimens of mammals, there is a finite time period within which to act before the sample decomposes. Determination of this “window of opportunity” to collect and preserve epididymal sperm would be of benefit for furthering research on methods of preservation and assisted reproductive technologies, especially for exotic animals.

To date, most laboratory animal research for in vitro fertilization (IVF) involves the use of epididymal sperm because of the difficulty in collecting an ejaculated sample. Early studies have reported fertilization (Iwamatsu and Chang, 1971) and embryo development (Miyamoto and Chang, 1972) with mouse epididymal sperm used for IVF. Trounson et al. (1989) achieved a 34% fertilization rate for mouse oocytes after injecting epididymal sperm under the zona pellucida. Fertilization (Ron-El et al., 1995) as well as embryo development to the blastocyst stage (Markert, 1983) have been achieved with mouse epididymal sperm by intracytoplasmic sperm injection (ICSI). Live offspring were reported after ICSI with mouse epididymal sperm (Roknabadi et al, 1994; Kimura and Yanagimachi, 1995). Normal mouse fetal development was reported after IVF with

epididymal sperm cooled to 4°C immediately after collection (Fuller and Whittingham, 1996) as well as with frozen-thawed epididymal sperm (Tada et al., 1994). Live offspring have also been produced by IVF with cryopreserved epididymal sperm from the mouse (Songsasen et al., 1997).

Blandau and Rumery (1964) compared fertilization rates between cauda and caput rat epididymal sperm and reported higher fertilization rates for cauda epididymal sperm when used for artificial insemination (AI). Live offspring were produced by intrauterine insemination (IUI) of frozen-thawed rat epididymal sperm (Nakatsukasa et al., 2001). The use of rat epididymal sperm in conjunction with IVF has resulted in fertilization (Blandau and Rumery, 1961), embryo development and live offspring (Toyoda and Chang, 1974). Moore and Akhondi (1996) reported that IVF capacity of rat sperm correlates with declining sperm velocity in a straight line (VSL, progressive motility) as assessed by computer-aided sperm analysis (CASA) and with the site of recovery from the epididymides. Sperm from the proximal cauda epididymidis exhibited a fertilization rate of 68% compared with 44 and 47% from samples collected from the distal cauda epididymidis and the vas deferens, respectively.

It has been reported that rat epididymal sperm when injected into germinal-vesicle stage oocytes would decondense but failed to produce pronuclei (Thadani, 1979). Dozortsev et al. (1998) have achieved fertilization (10%) in the rat using ICSI technology and cauda epididymal sperm. Live rat offspring have been produced with cryopreserved transgenic epididymal sperm used in conjunction with a modified ICSI technique (Piezo drilling) (Hirabayashi et al., 2002).

Fertilization was achieved by IVF with hamster epididymal sperm (Yanagimachi and Chang, 1964). Seshagiri and Bavister (1990) reported the production of offspring

from IVF with hamster epididymal sperm. Uehara and Yanagimachi (1976, 1977) reported the decondensation and formation of pronuclei of hamster epididymal sperm isolated from the testis, caput and cauda epididymidis and injected into hamster oocytes.

Higher rates of fetal development (Orgebin-Crist, 1969) and larger litter size (Paufler and Foote, 1968) in the rabbit after IUI with epididymal sperm was reported with cauda epididymal sperm and caput epididymal sperm. Further studies have reported successful IVF in the rabbit using ejaculated sperm for fertilization (Chang, 1959; Brackett and Williams, 1968) and live offspring (Chang, 1959) as well as embryo development to the blastocyst stage when using rabbit epididymal sperm for IVF (Ogawa et al., 1972; Hosoi et al., 1981). Brackett et al. (1978) compared rabbit cauda epididymal sperm to ejaculated sperm for IVF and reported that epididymal sperm (73.1%) had a higher fertilization rate than ejaculated sperm (36.6%). Embryo production in the rabbit using ICSI was reported with development to the 4-cell stage (Iritani et al., 1988). Hosoi and Iritani (1993) reported the birth of two normal rabbits after ICSI with fresh epididymal sperm. Higher blastocyst development (39%) and live offspring production (7 young) have been reported in the rabbit using the ICSI technique with ejaculated sperm (Deng and Yang, 2001).

In the mouse, efforts have been made to determine length of time (up to 7 days at 4°C and 24 hours at room temperature) before epididymal sperm collection may no longer be useful for the production of offspring (Christian et al., 1993; An et al., 1999a, 1999b; Stilley, 2002). Mouse pups (34%) have been produced by using IVF with cooled epididymal sperm stored for 7 days after death (Songsasen et al., 1998; An et al., 1999b; Kishikawa et al., 1999). Blastocyst development was achieved after IVF with mouse epididymal sperm harvested after 15 hours of storage at room temperature (Chrisitan et al.,

1993). Foote (2000) reported that a motile epididymal sperm could be obtained from rat testicles stored at 5°C for 24 hours postmortem. Yu and Leibo (2002) have reported that canine epididymal sperm maintained motility, membrane integrity as well as the ability to attach to the zona pellucida up to 8 days when stored in the epididymis at 4°C. One female pup was produced by AI with canine frozen-thawed epididymal sperm collected postmortem after preservation at 7°C long enough to be transported to the cryopreservation facility (Marks et al., 1994).

Since it has been found that viable epididymal sperm can be obtained from epididymides stored at 4 to 5°C for 24 hours postmortem, the objective of this experiment was to determine the postmortem “window of opportunity” to collect viable rat epididymal sperm from testicles stored at 4°C.

Materials and Methods

Experimental Design

Fifteen sexually mature male Sprague-Dawley (CD) rats were purchased from Charles River Laboratories (Raleigh, NC) and euthanized for use in this experiment. Each pair of testicles was assigned an identification number when processed. Testicles were randomly allotted to storage-time treatment groups. One testicle of each pair was assigned to the control storage time (24-hour cool storage-time treatment group) (n=15) at 4°C and the other testicle was then randomly allotted to one of three postmortem storage-time treatment groups (48 hours, or 72 hours or 96 hours) (n=5 for each group).

Experimental Procedure

At 10 weeks-of-age the rats were euthanized by CO₂ asphyxiation followed by cervical dislocation. It was previously determined that the method of euthanasia for rats does not affect sperm postmortem parameters (Slott et al., 1994). Each rat was then

weighed and body length was measured from nose to tip of the tail. The scrotal region was washed with 70% ethanol and sterile gauze. An incision was made into the caudal $\frac{1}{4}$ of the scrotum and each testicle was exteriorized with manual pressure. Another incision was made in the tunica vaginalis and the testicles were again exteriorized. Each testicle was removed from the body by cutting ~1 mm from the cranial edge of the caput epididymidis. Testes were then placed into a labeled plastic Petri dish and placed into storage at 4°C in a refrigerator.

At the allotted storage-time treatment period, each testicle was presented so that the corpus epididymidis was above the vas deferens. This placement allowed for assessment of the side of origination of the testicle. Each testicle was then measured for length from cranial to caudal pole and testicle circumference (including the corpus epididymidis). Weight of the testis was then determined before the epididymis was dissected away from the testis proper. The epididymis was individually weighed with and then without the cauda epididymidis.

Sperm TL medium (Parrish et al., 1988) was prepared with the addition of 0.006 g/ml of bovine serum albumin (Sigma, St. Louis, MO), 1mM sodium pyruvate and 25 µg/ml of gentamicin (GibcoBRL, Grand Island, NY) and warmed in a water bath to 39°C prior to use with the epididymal sperm.

Each cauda epididymidis was dissected free of fatty tissue, minced and then placed into a 15-mm, 24-well plastic Petri dish (Becton Dickinson & Company, Franklin Lakes, NJ) and rinsed with 500 µl of Sperm TL medium, as previously described by Tulsiani et al. (1993). The epididymis was allowed to sit for 5 minutes to allow sperm to swim out of the lumen of the cauda epididymidis. After co-incubation, the epididymal tissue was removed from the sperm solution and discarded. The sperm solution was then relocated to a 1.5-ml

amber microcentrifuge tube (Brinkmann, an Eppendorf Company, Westbury, NY) for further processing.

The percent progressively motile (PPM) sperm and the percent of sperm with an intact acrosome (PIA) for each sperm sample at each time period were evaluated by the same technician. At the allotted time, the rat epididymal sperm were then subjected to microscopic analysis for PPM using a Nikon Labophot 100X phase contrast light microscope (Nikon Instruments, Inc., Lewisville, TX). The PPM from each 10- μ l droplet sample was established by evaluating three fields under the microscope. The total number of sperm obtained from the minced cauda epididymidis during the swim out was determined with a 1:20 dilution created by placing 5 μ l of the sperm suspension into 95 μ l of Sperm TL medium. A 10- μ l sample of this sperm suspension was placed on each side of a hemacytometer slide and allowed to settle before evaluation. The number of sperm counted in five of the squares was averaged from both sides and that number value indicated the number $\times 10^6$ of sperm/ml. Since the sample's total volume was 500 μ l, the number of sperm counted was divided by 2 to determine the final sperm concentration.

The percent of viable sperm was determined when 20 μ l of the original sperm suspension was added to 10 μ l of eosin B/fast green stain (Aalseth and Saacke, 1986). The sperm mixture was thoroughly mixed and 10 μ l was pipetted onto a clean microscope slide. The solution was spread over the slide and then heat dried to fix the sperm cells. The slide was examined under oil at 400X using a light microscope and 200 sperm were counted. When the sperm excluded the dye they were considered to have maintained the sperm plasma membrane and acrosomal membrane integrity, and were recorded as having an intact acrosome. Those epididymal sperm that had absorbed the dye (appearing red)

were considered membrane compromised and therefore, nonviable (Aalseth and Saacke, 1986).

Statistical Analysis

Results from this study are expressed as mean values \pm SEM for testicular weight, circumference and length and including epididymal weight and length. Mean percentages were also used to express sperm parameters, such as PPM and PIA. Data were analyzed using a simple one-way ANOVA in SAS[®] (Version 8.0) with a Tukey multiple comparison test. The comparisons were made among the control samples (24 hours) and the treatment samples from the different storage times starting at 48 through 96 hours postmortem. Sperm parameters were analyzed with testicular measurements as the covariate to determine if differences were influenced by side of testicular origin.

Results

Mean body weight and body length measurements for the male rats are included in Table 2.1. No significant differences ($P>0.05$) were detected among any of the body measurements evaluated in this study.

The mean testicular values for testicular weight, length, circumference, epididymal weight and the approximate number of sperm obtained per cauda epididymidis from each storage-time treatment group are presented in Table 2.2. There was no significant difference ($P>0.05$) noted among any of the paired testicle measurements. The mean (\pm SE) total number of sperm collected per cauda epididymidis in each cool storage-time treatment group ranged from 11.8 ± 2.4 to $21.4\pm7.1 \times 10^6$.

The PPM for epididymal sperm had a significant decline ($P<0.01$) from 24 to 48 through 96 hours of cold storage (46.0 ± 2.5 , 28.0 ± 3.7 , 18.0 ± 3.4 and $20.0\pm4.5\%$,

Table 2.1. Mean (\pm SEM) body weight and length of mature, male Sprague-Dawley rats shortly after euthanasia

Trt Group	n	Body Weight (kg)	Body Length (cm)
24 h	15	0.365 \pm 0.02	42.5 \pm 1.9
48 h	5	0.367 \pm 0.03	42.7 \pm 1.5
72 h	5	0.362 \pm 0.02	41.2 \pm 2.8
96 h	5	0.365 \pm 0.01	43.6 \pm 1.7

Means in columns were not significantly different ($P>0.05$).

Table 2.2. Mean (\pm SEM) testicular values from testes of mature rats collected postmortem and stored at 4°C at 24-, 48-, 72- and 96-hour intervals

Trt Group	Testicle Weight (g)	Testicle Length (cm)	Testicle Circum.* (cm)	Epididymal Weight (g)	Cauda Weight (g)	No. Sperm [†] ($\times 10^6$)
24 h	2.2 \pm 0.04	2.2 \pm 0.1	3.8 \pm 0.06	0.81 \pm 0.02	0.14 \pm 0	17.4 \pm 2.7
48 h	2.3 \pm 0.08	2.0 \pm 0.1	3.7 \pm 0.05	0.85 \pm 0.03	0.15 \pm 0.01	21.4 \pm 7.1
72 h	2.2 \pm 0.07	2.2 \pm 0.1	3.8 \pm 0.07	0.78 \pm 0.04	0.12 \pm 0.01	11.8 \pm 2.4
96 h	2.2 \pm 0.06	2.4 \pm 0.1	3.8 \pm 0.17	0.81 \pm 0.04	0.14 \pm 0	18.8 \pm 2.9

*Circum. = circumference.

[†] No. Sperm = mean total number of sperm harvested per cauda epididymidis.

Means in columns were not significantly different ($P>0.05$).

respectively) (Figure 2.1). Mean percent of sperm with intact acrosomal membranes (PIA) from rat epididymal sperm samples were not significantly different for 24, 48, 72 and 96 hours of storage at 4°C (50.1 ± 3.3 , 48.6 ± 9.7 , 50.6 ± 7.5 and $32.8 \pm 3.3\%$, respectively).

Discussion

This experiment was a pilot study used to assess the length of time that postmortem storage of sperm in the epididymis would maintain the viability of rat epididymal sperm. Also, to determine if the pattern of sperm viability was similar to those previously observed in this laboratory for both the mouse and the dog (Stilley et al., 1999, 2000; Stilley, 2002) when the same cooling conditions were applied to the testicles of the laboratory rat.

In this study with rat epididymal sperm, an initial decline was noted for PPM from 24 to 48 hours of cool storage followed by a plateau in PPM to 96 hours. The decrease in motility from 46% at 24 hours to 20% at 96 hours was comparable to the reported values in the mouse of 43% at 24 hours to 16% at 96 hours in a study from this station (Stilley, 2002).

Christian et al. (1993) monitored PPM values for mouse epididymal sperm harvested from testes that had been stored at room temperature (25°C) following death for up to 24 hours. At 20 to 24 hours the PPM for mouse epididymal sperm at 25°C was 20 to 30%, expectedly lower values than those reported for both mouse and rat epididymal sperm stored at cooler temperatures (4°C). The mouse epididymal sperm harvested after 15 hours at 25°C (50 to 80% PPM) was used for IVF and produced 20% blastocysts.

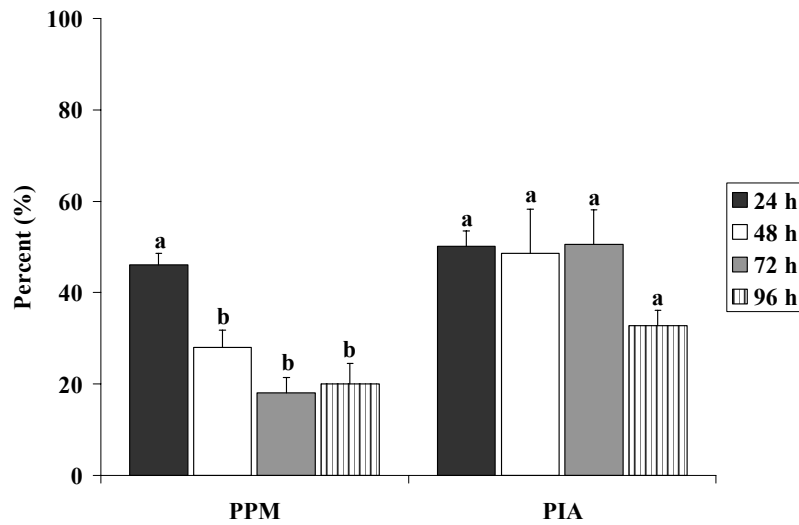


Figure 2.1. Mean (\pm SEM) percent progressive motility (PPM) and mean percent membrane integrity (PIA) for rat epididymal sperm stored in the cauda epididymidis of testes at 4°C for 24, 48, 72 or 96 hours (h). ^{a,b}Means with different superscripts within sperm parameters are significantly different ($P < 0.01$).

Fuller and Whittingham (1996) reported mouse epididymal sperm progressive motility values of 19 to 75% when sperm were cooled to 4°C over a 4-hour period outside of the testicle. When these cooled mouse sperm were exposed to mature ova, resulting embryos developed into normal fetuses at a significantly higher percentage (63.9 to 74.1%) than with fresh sperm (53.8%). These mouse epididymal sperm values were similar to the progressive motility exhibited by rat sperm after storage in the testicle for 24 through 96 hours (46 to 20%) in this experiment.

The PPM values reported for rat epididymal sperm in our study were lower (46 to 20%) than that reported for fresh rat cauda epididymal sperm collected immediately after death (75%) (Nakatsukasa et al., 2001). Progressive motility of 46% at 24 hours in our study, however, was greater than the progressive motility that Nakatsukasa et al. (2001) reported for frozen-thawed rat epididymal sperm of only 9%. In the latter study, intrauterine insemination (IUI) with either fresh or frozen rat epididymal sperm produced live offspring.

Kishikawa et al. (1999) reported that epididymal sperm harvested from mice at 10 days postmortem had 30% progressive motility and could be used for ICSI, with an 80% fertilization rate. Furthermore, epididymal sperm collected after 20 days postmortem and used with ICSI produced live offspring. Thus, rat sperm cooled at 4°C in the testis for 24 hours could be used for assisted reproductive technologies (e.g., AI, IVF and ICSI).

Bezuidenhout et al. (1995) evaluated the PPM of four different exotic animals (African buffalo, Red hartebeest, eland and Burchell's zebra) after being stored postmortem at 4°C in the testicle. The epididymal sperm motility exhibited by the African buffalo throughout the study was similar to the sperm motility pattern found with the rat in the present experiment. The Red hartebeest, eland and Burchell's zebra had a much

shorter duration for epididymal sperm motility when stored at 4°C in the epididymis compared with the African buffalo.

In contrast to PPM values in the present study, the mean PIA values were not different among any of the storage-time treatment groups from 24 (50%) through to 96 hours (33%) postmortem. This finding was comparable to those of Stilley (2002), who assessed mouse epididymal sperm exposed to cool storage during a similar postmortem interval. In the latter study, mouse epididymal sperm PIA values were 53, 60, 47, 44 and 25% for 0, 24, 48, 72 and 96 hours postmortem, respectively. Stilley (2002) (testicles stored at 4°C) and Christian et al. (1993) (testicles stored at room temperature) both reported PIA values of 60% for 24 hours postmortem for mouse epididymal sperm, which are slightly higher than the PIA values of 50% reported for male rats in the present study.

The frozen-thawed rat epididymal sperm used to produce offspring by IUI, had PIA values of $\leq 9\%$ (Nakatsukasa et al., 2001), which was lower than any of the PIA values noted for rat sperm in our study. Rat sperm collected from the proximal cauda epididymidis that had been stored at 4°C, exhibited progressive motility for the first 24 hours and then declined 20% during the subsequent 24-hour intervals up to 96 hours postmortem. These same epididymal sperm gave all indications of maintaining membrane integrity up to 96 hours postmortem after staining. The pattern found for rat epididymal sperm parallels the decline in PIA noted for both the mouse and the dog epididymal sperm reported in recent studies by this laboratory (Stilley, 2002).

Moore and Akhondi (1996) have stated that a decline in sperm VSL over a 5-hour incubation period was highly correlated with IVF outcome in the rat. Unfortunately, the authors did not assess the membrane integrity of the rat epididymal sperm used for IVF. In our study, rat epididymal sperm had a decrease in the progressive motility over time in

cool storage. This could indicate a potential decrease in sperm fertility, however, it should not be overlooked that the maintenance of membrane integrity could also indicate that fertilizing ability of the rat sperm has been preserved.

Conclusion

This pilot experiment showed that there was a decline in the pattern to survivability of rat epididymal sperm retrieved from the cauda epididymidis of the testis stored at 4°C for up to 96 hours. This study also demonstrated that the pattern over time reported herein was similar to the pattern over time reported for both the mouse and the dog epididymal sperm in studies by this laboratory. With this information, we propose that it is possible that this same pattern for cooled epididymal sperm could be found in farm animals. Future experiments should be completed in domestic mammalian species, as well as with closely related endangered species, to aid in the preservation of their gametes. It is recommended that further investigation of epididymal sperm be undertaken in commercial livestock species. These types of experiments would help to further develop assisted reproductive technologies that could keep some of the more valuable species alive for future generations to enjoy.

CHAPTER 3

PRESERVATION OF CAPRINE EPIDIDYMAL SPERM STORED IN THE EPIDIDYMIDES OF TESTES AT 4°C FOR 24, 48, 72 AND 96 HOURS

Introduction

Epididymal sperm has become an important part of current reproductive research efforts toward the preservation of endangered and exotic species. Epididymal sperm is a valuable means of preserving genetic material because of the ease of collection from males that have been culled or that have prematurely died. In the case of exotic species, the gametes could be collected from a genetically valuable specimen at postmortem. The most efficient way to conserve the germplasm of endangered species would be to perfect the method using domestic models prior to extending the techniques to endangered animals.

Mouse epididymal sperm first produced fertilized zygotes for Iwamatsu and Chang (1971) followed by fertilization and embryo development when used for in vitro fertilization (IVF) (Miyamoto and Chang, 1972). Fuller and Whittingham (1996) reported the production of normal mouse fetuses after IVF with epididymal sperm that had been cooled to 4°C immediately after collection. One early report described the use of rat epididymal sperm for IVF embryo development and the birth of 43 live offspring (Toyoda and Chang, 1974).

Blastocysts have been produced from IVF with fresh caprine epididymal sperm (Song and Iritani, 1988; Blash et al., 2000). Graff et al. (1996) reported two ongoing pregnancies from IVF-produced embryos using fresh bovine epididymal sperm. Cryopreserved caprine epididymal sperm was used to produce a live kid by artificial insemination (AI) (Blash et al., 2000). Foote (2000) in a letter to the editor indicated that a live calf had been born by AI using bovine epididymal sperm. Barker and Gandier (1957)

also reported the birth of a foal produced from AI with frozen-thawed equine epididymal sperm. Bartels et al. (2001) reported the live birth of an eland produced by AI with frozen-thawed epididymal sperm.

Studies have also reported the use of epididymal sperm for IVF in a few exotic species, such as the lion (Jewgenow et al., 1997; Bartels et al., 2000), puma (Jewgenow et al., 1997), blesbok (Winger et al., 1997) and African buffalo (Shaw et al., 1995). Meintjes et al. (1997) also reported the use of Burchell's zebra epididymal sperm for IVF with both Burchell's and Hartmann's zebra oocytes and the production of blastocysts.

Researchers have attempted to determine the time constraints for collecting viable epididymal sperm from various species including mice (Kishikawa et al., 1999), rats (Chapter 2), cats (Pushett et al., 2000), dogs (Stilley et al., 1999, 2000; Graff et al., 2000; Yu and Leibo, 2002), goats (Song and Iritani, 1988; Pangestu, 1997; Anel et al., 2000) and cattle (Foote, 2000). Studies on harvesting epididymal sperm postmortem have also been reported on exotic species including the Sika deer (Hishinuma et al., 2003), African buffalo (Bezuidenhout et al., 1995; Friedmann et al., 2000), zebra (Bezuidenhout et al., 1995; Lubbe et al., 2000), Red hartebeest (Bezuidenhout et al., 1995) and the eland (Bezuidenhout et al., 1995). Most of these studies found that viable epididymal sperm could be harvested at postmortem for potential use in the production of offspring using assisted reproductive technologies. Each of these species had variation in the amount of time the sperm could remain viable within the testes prior to the harvesting process.

Another variable affecting the harvest of the maximum number of viable sperm per animal was the method of testes storage. Most of these species provided a greater number of viable sperm when stored in the epididymis at 4 to 5°C prior to collection compared with testicles stored at an elevated temperature.

Previous experiments have found that male laboratory rats can provide viable epididymal sperm up to 96 hours postmortem when the testes are maintained at 4°C (Chapter 2). With increased research interest in epididymal sperm, preservation methods need to be developed that can further increase the utility of the sperm harvested. Blash et al. (2000) reported that epididymal sperm collected immediately at postmortem from the goat can be successfully cryopreserved. Epididymal sperm recovered <6 hours postmortem, or at castration from Sika deer (Hishinuma et al., 2003), African wild ruminants (Kilian et al., 2000), domestic stallions, zebra and rhinoceros (Lubbe et al., 2000), as well as dogs (Stilley et al., 2000) and cats (Pushett et al., 2000) can be preserved by cryopreservation with minimal loss of progressive motility. Stilley et al. (2000) also reported that canine epididymal sperm collected beyond 6 hours postmortem displayed adequate survivability after cryopreservation. The use of cryopreserved epididymal sperm for IVF in exotic species has been reported in animals, such as the blesbok (Winger et al., 1997) and puma and lion (Jewgenow et al., 1997).

Most researchers working with endangered species currently working on assisted reproductive technology methods to aid in animal species preservation use related domestic bovine, caprine and equine species as animal models. Thus, one objective of this study would be to determine the “window of opportunity” for collection of epididymal sperm to create a research model for exotic species that are closely related to the farm animal species. The second objective of this study would be to determine if the cryopreservation technique utilized for preservation of ejaculated sperm was applicable for preserving postmortem epididymal sperm collected from domestic caprine males.

Materials and Methods (Cool Storage)

Experimental Design (Cool Storage)

Testicles of caprine males were collected as pairs and shipped by overnight to the laboratory for further processing. Upon arrival, one of the testicles was stored at 4° C for 48 hours, 72 hours or 96 hours postmortem (n=8, 8 and 7 per cool storage-time treatment group, respectively) while the other testicle (control) of a pair was immediately processed (24-hour cool storage-time treatment group) (n=23). The percent progressively motile (PPM) and percent intact acrosomes (PIA) were observed immediately after processing by the same technician for each sample at each cool storage-time period.

Experimental Procedure (Cool Storage)

Caprine testicles were harvested as pairs from mature bucks at an abattoir in San Angelo, TX. Each pair of collected testicles was transported overnight to the Louisiana State University Embryo Biotechnology Laboratory individually packaged in Ziploc[®] bags, placed on ice to cool to 4° C and shipped in a Styrofoam Igloo[™] container by FedEx. For each cool storage-time treatment group the randomly allotted testicles were processed in the following manner. Testicles were allowed to warm to room temperature (25°C) from cool storage (4°C) for ≤30 minutes. Testicles were dissected away from the tunica vaginalis and other extraneous tissues during the warming period.

Each testicle was situated so that the corpus epididymidis was above the vas deferens to determine the side of origin for each testicle. Testicle length was measured from proximal (cranial) to distal (caudal) pole and included the end of the cauda epididymidis. Circumference was measured midway between the ends of the caput epididymidis and the cauda epididymidis and also included the attached corpus epididymidis on each testicle.

The epididymis was measured by following the external curvature with a string that was then referenced against a ruler to determine length. A positive correlation has been reported between IVF fertility and epididymal length in men with congenital absence of the vas deferens (Patrizio et al., 1994) but this correlation has not been reported for morphologically normal males. The testis along with the epididymis was weighed on a balance scale (American Family, Inc.). The epididymis was then carefully dissected away from the testis. Once free of the testis, the epididymis was sectioned and weighed in three segments (caput, corpus and cauda) using a Mettler Toledo metric scale (Model #AE 50, Columbus, OH).

Prior to use as the extending medium for the caprine epididymal sperm, Sperm TL medium (Parrish et al., 1988) was prepared with the addition of 0.006 g/ml of bovine serum albumin (Sigma, St. Louis, MO), 1mM sodium pyruvate (Sigma) and 25 µg/ml of gentamicin (GibcoBRL, Grand Island, NY). After addition of final components the Sperm TL medium was warmed to 39°C in a water bath.

After measuring the physical parameters of the epididymis it was minced and placed into a 60-mm plastic Petri dish (Becton Dickinson & Company, Franklin Lakes, NJ). The minced epididymis was rinsed with 3 ml of previously warmed Sperm TL medium and allowed to incubate with the medium for 5 minutes. After the 5-minute incubation, the epididymal sperm solution was collected and placed in a 15-ml plastic centrifuge tube (Corning, Inc., Corning, NY). The epididymal sperm solution was then centrifuged (Sorvall® Tabletop Refrigerated Centrifuge RT-6000, Sorvall® Centrifuges, Wilmington, DE) at 700 x g for 6 minutes to create a pellet of epididymal sperm. The supernatant was aspirated and discarded and the pellet was subsequently resuspended in

fresh Sperm TL medium. A 10- μ l volume sample of epididymal sperm suspension was removed for analysis.

Sperm analysis included PPM, PIA and a count to determine the total number of sperm obtained per cauda epididymidis. A 5- μ l volume, of the 10- μ l volume sample removed from the epididymal sperm suspension, was added to 95 μ l of Sperm TL medium for PPM and PIA analysis. The remaining 5 μ l was added to 95 μ l of distilled water to create a 1:20 dilution for a hemacytometer count. The PPM was evaluated with the 10- μ l solution of the 1:20 sperm solution:Sperm TL medium dilution using a 100X phase contrast microscope (Nikon Labophot, Nikon Instruments, Inc., Lewisville, TX).

A microscope slide with a sample of eosin B/fast green stained epididymal sperm was used to evaluate PIA. The eosin B/fast green stained epididymal sperm sample was created by using 20 μ l of the 1:20 sperm solution and mixing it with a previously prepared 10- μ l sample of eosin B/fast green stain (Aalseth and Saacke, 1986). After co-incubating for 1 minute a 10- μ l drop of the mixture was thinly spread on the slide surface and the epididymal sperm were heat fixed. Each sample was evaluated by counting 200 sperm and the respective acrosomal status of each caprine epididymal sperm was determined. Epididymal sperm and their respective acrosomes were categorized as intact if both internal and external membranes retained their integrity and therefore, excluded the dye. Those sperm that appeared pink or red were considered membrane compromised and therefore, not acrosome intact.

Statistical Analysis (Cool Storage)

Results of this study are expressed as a mean value \pm SEM for testicle morphological parameters (e.g., testicular weight, length and circumference as well as

epididymal weight and length) and sperm parameters (e.g., percent progressive motility and percent of sperm with an intact acrosome). Data analysis was performed using simple one-way ANOVA in SAS[®] (Version 8.0) with a Tukey multiple comparison adjustment. Testicular and epididymal measurements were used as covariates in the analysis of PPM and PIA to determine if differences among cool storage-time treatment groups were due to differences among individual goat testicles.

Results (Cool Storage)

Testicular Parameters

Caprine mean values for testicular weight, length, circumference, epididymal length, cauda weight and number of sperm obtained from the cauda epididymides in each storage-time treatment group are presented in Table 3.1. Total mean sperm number collected per cauda epididymidis ranged from 1.5×10^9 to 0.7×10^9 for the cool storage-time treatment groups. There was a downward trend over time but no significant difference in numbers of sperm collected. There was no significant difference ($P>0.05$) detected among any of the paired testicle measurements.

Parameters for Sperm Stored in the Cauda Epididymidis at 4°C

Mean PPM for caprine epididymal sperm at 24, 48, 72 and 96 hours of cool storage was: 68.3 ± 1.5 , 55.7 ± 2.8 , 46.3 ± 2.8 and 41.9 ± 2.7 , respectively (Figure 3.1). A significant decline ($P<0.01$) was noted from 48 to 72 hours and again from 72 to 96 hours of cool storage.

Mean PIA for caprine epididymal sperm at 24, 48, 72 and 96 hours of cool storage at 4°C was 66.5 ± 1.6 , 55.4 ± 2.6 , 56.8 ± 4.8 and 48.4 ± 3.5 , respectively (Figure 3.1). An initial decrease ($P<0.01$) in PIA of epididymal sperm occurred from the 24-hour to the 48-hour

Table 3.1. Mean (\pm SEM) testicular values from caprine testes collected postmortem and stored at 4°C for 24, 48, 72 and 96 hours

Trt Group	Testicle Weight (kg)	Testicle Length (cm)	Testicle Circum.* (cm)	Epididymal Length (cm)	Cauda Weight (g)	No. Sperm [†] ($\times 10^9$)
24 h	0.126 \pm 0.01	12.2 \pm 0.2	15.4 \pm 0.3	22.7 \pm 0.3	4.2 \pm 0.19	1.5 \pm 0.2
48 h	0.109 \pm 0.01	11.7 \pm 0.4	14.4 \pm 0.5	21.5 \pm 0.6	3.9 \pm 0.26	0.9 \pm 0.1
72 h	0.141 \pm 0.01	12.5 \pm 0.2	15.7 \pm 0.2	23.6 \pm 0.4	4.2 \pm 0.37	0.9 \pm 0.3
96 h	0.150 \pm 0.01	12.3 \pm 0.3	15.5 \pm 0.3	21.9 \pm 0.6	4.2 \pm 0.20	0.7 \pm 0.2

*Circum. = circumference.

[†] No. Sperm = mean total number of sperm harvested per cauda epididymidis.

Means were not significantly different ($P > 0.05$).

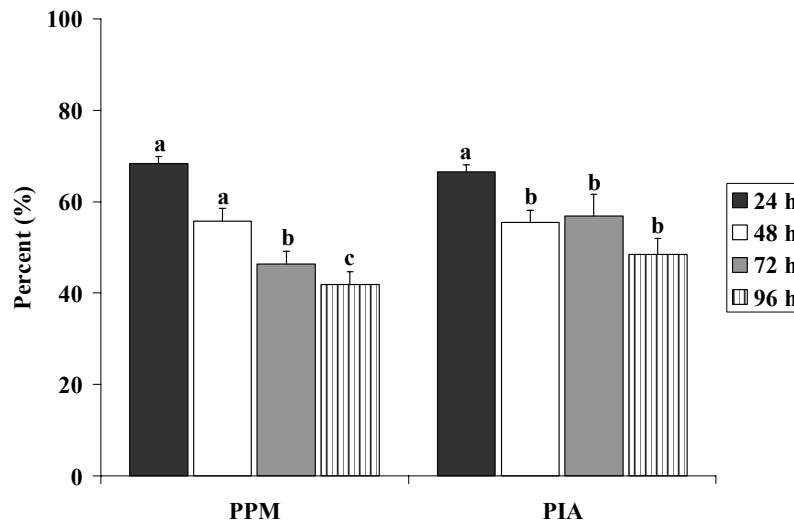


Figure 3.1. Mean (\pm SEM) percent progressive motility (PPM) and mean percent membrane integrity (PIA) for caprine epididymal sperm stored in the cauda epididymidis of testes at 4°C for 24, 48, 72 or 96 hours (h). ^{a,b,c}Means with different superscripts within sperm parameters are significantly different ($P < 0.01$).

cool storage-time treatment groups. The mean PIA decreased and reached a plateau at 48 hours and remained relatively constant through to 96 hours of cool storage.

Materials and Methods (Cryopreservation)

Experimental Design (Cryopreservation)

Cryopreserved caprine epididymal sperm samples were collected all through the experiment described in the Cool Storage section. Pairs of goat epididymides that produced 2 or more straws (7.5×10^6 sperm/straw) for liquid nitrogen (LN₂) storage from testicles stored during the 24-, 48-, 72- and 96-hour cool storage-time treatment groups (n=23, 8, 8 and 7 per cool storage-time treatment group, respectively) were included in this experiment. Cryopreserved samples of epididymal sperm were thawed and analyzed for PPM and PIA at 0 and 3 hours post-thaw, for each cool storage-time treatment group of 24, 48, 72 and 96 hours.

Experimental Procedure (Cryopreservation)

The cryoprotective medium used for preservation of caprine epididymal sperm was prepared from egg-yolk citrate with 7% glycerol (EYG) (Salisbury et al., 1941). This medium was prepared in one batch and frozen for later use in 10-ml aliquots. On day of use, EYG was thawed at 4°C until liquidified and then warmed to room temperature (25°C) in a 15-ml plastic conical tube (Corning, Inc., Corning, NY).

When PPM and the total number of sperm were determined (as previously described in the Cool Storage section) then the remaining epididymal sperm sample was cryopreserved. Each sperm sample was centrifuged (700 x g) for 6 minutes to aid in the removal of the Sperm TL medium supernatant. Part A (0% glycerol) of EYG extender was added to the epididymal sperm pellet to create a 30×10^6 sperm/ml dilution. This diluted

sperm sample was cooled at 4°C for a total of 1 hour. During the 1-hour cooling period, additions of part B (14% glycerol) EYG made at 15-minute intervals. Each addition of part B EYG equaled 0.125% of the total final volume bringing the epididymal sperm concentration to 15×10^6 sperm/ml of medium. After 1 hour of equilibration and the addition of part B of the EYG extender, the sperm solution was drawn into 0.5-ml plastic straws (Cassou straw, IMV Technologies, Minneapolis, MN) and placed into the freezing chamber.

The freezing chamber was a rectangular Styrofoam[™] shipping container that was fitted with a metal 0.5-ml straw stand and a thermometer (Sensortek Model Bat-12, Costa Mesa, CA). The straw stand was a modified metal pipette rack that was 5 cm high. This rack only came into contact with the semen straws at two points, one at each end. The thermometer was first inserted through the side wall of the container and then into a straw filled with the cryoprotective medium. The thermometer was placed in the straw at this level to measure temperature changes in the LN₂ vapors at the level of the semen straws. The Styrofoam[™] container was filled with LN₂ to a height of 3 cm, allowing for a 2-cm space between the liquid and the straws.

The straws were placed 2 cm above the level of the LN₂ where the “start” temperature was -110°C. The straws of caprine epididymal sperm remained at this level for 8 minutes until they reached -100°C, and then they were directly immersed into the LN₂ (-196°C). The rate of freezing was -13°C/minute for the caprine epididymal sperm from 4°C to -100°C, as measured by the thermometer. Leboeuf et al. (2000) described a similar method for cryopreserving ejaculated goat sperm. The authors also stated that bull sperm could be successfully frozen using sperm cryopreservation protocols, such as that described above.

Caprine epididymal sperm were cryopreserved for ≥ 24 hours prior to thawing for analysis. Prior to post-thaw analysis, Sperm TL medium was prepared (as previously described in the Cool Storage section) and then warmed to 39°C. Straws of caprine epididymal sperm were removed from the LN₂ and placed into a 39°C water bath for 1 minute. The sperm samples were then emptied into a 15-ml centrifuge tube with 3 ml of pre-warmed Sperm TL medium. The epididymal sperm mixture was centrifuged (700 x g for 6 minutes) to aid in removal of the cryoprotectant. The sperm pellet was resuspended with another 3 ml of Sperm TL medium and was centrifuged once more. This process further diluted the cryoprotective medium away from the epididymal sperm. Sperm pellets were resuspended in 2 ml of Sperm TL medium and the frozen-thawed caprine epididymal sperm were then prepared for analysis.

In this experiment, PPM and PIA were evaluated at 0 and 3 hours post-thaw. Ritar and Salamon (1982) used 6 hours of extended culture at 37°C to assess the cryosurvivability of caprine sperm. Gidson and Graham (1969), Linford et al. (1976) and Kjaestad et al. (1993) reported a positive correlation for frozen-thawed bull sperm and fertility by assessing PPM at 0 hours of post-thaw. The 3-hour analysis of the PPM, as well as the PIA, has also been correlated with frozen bull sperm fertility (Saacke, 1970; Fleming et al., 1976; Chandler et al., 1978).

Statistical Analysis (Cryopreservation)

Results of this experiment are expressed as a mean value for caprine epididymal sperm parameters (e.g., PPM and PIA at prefreeze, 0 and 3 hours post-thaw). Data were analyzed using Proc Mixed in SAS[®] (Version 8.0) (SAS, 1996) and a repeated measures design, with a Tukey multiple comparison. This analysis determined if there was a

difference among 4°C cool storage-time treatment groups by examining the difference between the prefreeze and post-thaw PPM and PIA values.

Results (Cryopreservation)

Epididymal Sperm PPM

Mean prefreeze PPM for caprine epididymal sperm at 24, 48, 72 and 96 hours of cool storage was 68.3 ± 1.6 , 55.7 ± 2.8 , 46.3 ± 2.6 and $41.9 \pm 2.6\%$, respectively (Figure 3.2). A decrease ($P < 0.0334$) in sperm progressive motility occurred from 24 to 48 hours, however, at 48 through to 96 hours of cool storage the PPM values were not significantly different. Analysis of caprine epididymal sperm that was cryopreserved had mean PPM values for 24, 48, 72 and 96 hours of cool storage at 0 hour post-thaw of 18.7 ± 3.4 , 20.0 ± 6.2 , 25.6 ± 5.8 and $36.3 \pm 5.8\%$, respectively. No difference was detected among the cool storage-time treatment groups for PPM at 0 hour post-thaw. The post-thaw analysis made after 3 hours of incubation had mean PPM values for 24, 48, 72 and 96 hours of cool storage of 7.4 ± 3.0 , 12.9 ± 5.5 , 16.3 ± 5.1 and $24.4 \pm 5.1\%$, respectively. As with the values for 0-hour measurement, no difference was detected among the mean values for PPM for any of the cool storage-time treatment groups at 3 hours post-thaw.

Epididymal Sperm PIA

A difference in prefreeze PIA was noted between the 24- and the 96-hour cool storage-time treatment groups ($P = 0.0023$) (Figure 3.3). No difference is detected among the 24-hour to the 72-hour or the 48-hour to the 96-hour cool storage-time treatment groups (66.5 ± 2.0 , 55.4 ± 3.5 , 56.8 ± 3.3 and $48.4 \pm 3.3\%$ mean PIA for 24, 48, 72 and 96 hours of cool storage, respectively). The mean PIA values for cryopreserved samples at 0 hour post-thaw for the 24-, 48-, 72- and 96-hour cool storage-time treatment groups were:

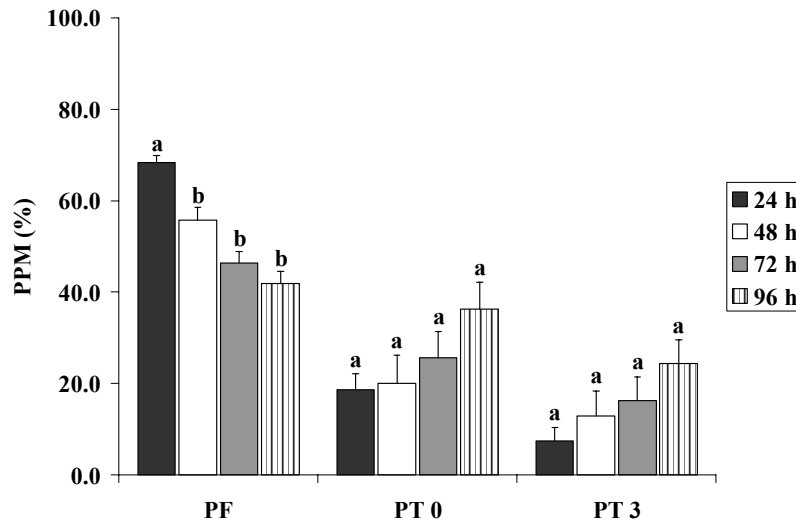


Figure 3.2. Mean (\pm SEM) percent progressive motility (PPM) at prefreeze (PF) and 0 and 3 hours post-thaw (PT) from cryopreserved caprine epididymal sperm stored in the cauda epididymidis of testes at 4°C for 24, 48, 72 or 96 hours (h). ^{a,b}Means with different superscripts within time of measurement are significantly different ($P < 0.0334$).

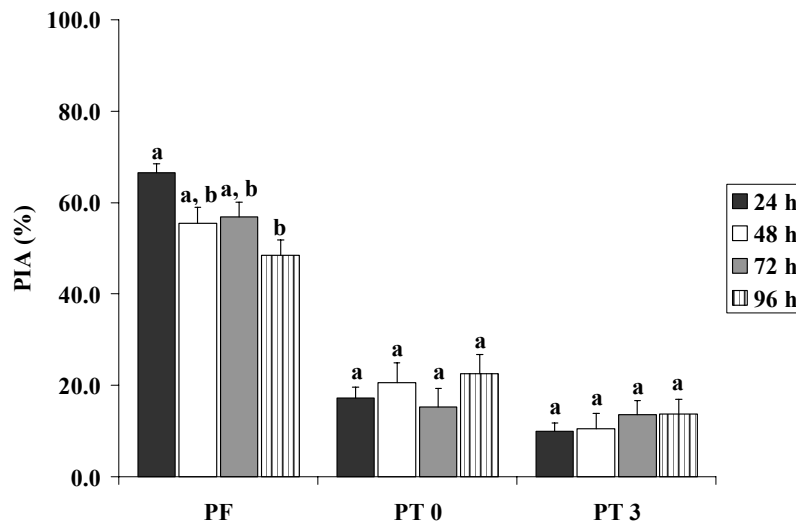


Figure 3.3. Mean (\pm SEM) percent with intact acrosomes (PIA) at prefreeze (PF) and 0 and 3 hours post-thaw (PT) from cryopreserved caprine epididymal sperm stored in the cauda epididymidis of testes at 4°C for 24, 48, 72 or 96 hours (h). ^{a,b}Means with different superscripts within time of measurement are significantly different ($P = 0.0023$).

17.2±2.4, 20.6±4.4, 15.3±4.1 and 22.6±4.1%, respectively. No difference was detected among the cool storage-time treatment groups for PIA at 0 hour post-thaw. The mean PIA values at 3 hours post-thaw were 10.0±1.8, 10.6±3.3, 13.6±3.1 and 13.8±3.1% for 24, 48, 72 and 96 hours of cool storage, respectively. Also, as with the values for 0-hour measurements, no difference was detected among the mean values for PIA for any of the cool storage-time treatment groups at the 3-hour measurements.

Significant differences were detected in mean PPM from prefreeze to thaw in all storage-time treatment groups (Figure 3.4), except for the 96-hour cool storage-time treatment group. Cryopreserved samples that had been previously stored in the testicle for 24 and 48 hours demonstrated a significant difference among the prefreeze and the 0-hour and the 3-hour post-thaw mean PPM values ($P<0.0001$). Only those cryopreserved samples from the 24-hour cool storage-time treatment group were found to have significant differences ($P=0.0004$) between the mean PPM values for 0 hour and 3 hours post-thaw.

Differences were also detected from prefreeze to post-thaw for mean PIA values in all storage-time treatment groups confirming the loss due to cryodamage (Figure 3.5). Differences were noted between prefreeze and 0-hour post-thaw mean PIA values for the cryopreserved samples from all cool storage-time treatment groups 24, 48, 72 and 96 hours of cool storage ($P<0.0001$ for the 24-hour through the 72-hour cool storage-time treatment groups and $P=0.0004$ for the 96-hour cool storage-time treatment group). All storage-time treatment groups for 24 through 96 hours of cool storage also had a significant decline ($P<0.0001$) between the prefreeze and the 3-hour post-thaw mean PIA values.

Discussion

The preliminary results from the mouse (Kishikawa et al., 1999; Stilley, 2002), the rat (Chapter 2) and the male dog (Stilley et al., 1999, 2000) have similar epididymal sperm

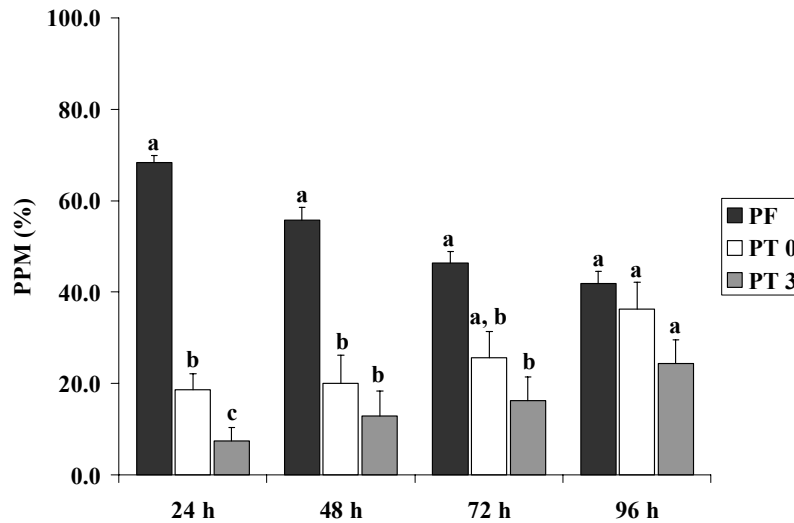


Figure 3.4. Mean (\pm SEM) percent progressive motility (PPM) at preefreeze (PF) and 0 and 3 hours post-thaw (PT) from cryopreserved caprine epididymal sperm stored in the cauda epididymidis of testes at 4°C for 24, 48, 72 or 96 hours (h). ^{a,b}Means with different superscripts within storage-time treatment groups are significantly different ($P < 0.0001$). ^{b,c}Means with different superscripts within storage-time treatment groups are significantly different ($P = 0.0004$).

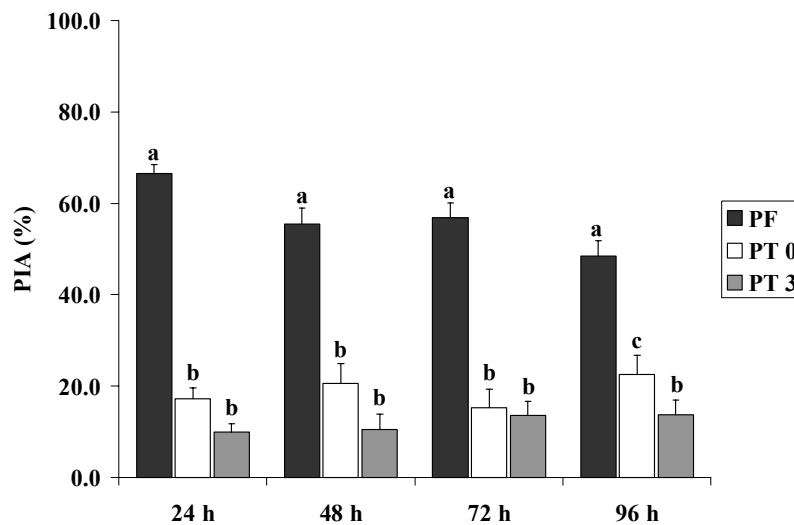


Figure 3.5. Mean (\pm SEM) percent with intact acrosomes (PIA) at preefreeze (PF) and 0 and 3 hours post-thaw (PT) from cryopreserved caprine epididymal sperm stored in the cauda epididymidis of testes at 4°C for 24, 48, 72 or 96 hours (h). ^{a,b}Means with different superscripts within storage-time treatment groups are significantly different ($P < 0.0001$). ^{a,c}Means with different superscripts within storage-time treatment groups are significantly different ($P = 0.0004$). ^{c,b}Means with different superscripts within storage-time treatment groups are not significantly different.

parameter patterns noted for prefreeze storage-time treatment group in this experiment with goat testicles. The domestic caprine males exhibited a gradual decline in epididymal sperm PPM and PIA when measured after cool storage within the cauda epididymidis at 4°C for up to 96 hours postmortem.

All mean testicular and epididymal parameters measured in the 48-hour cool storage-time treatment group exhibited a trend for lower weights and lengths when compared with the testicles allotted to the control and other cool storage-time treatment groups, however, these parameters were not significantly different. These were statistically analyzed as covariates for PPM and PIA and no significant differences were detected. Unlike the positive correlation between epididymal length and fertility reported by Patrizio et al. (1994) for the human male, this study found no correlation in the male goat between length of the epididymis with PPM or PIA values, which are commonly used to estimate fertility. Thus, the differences noted for the PPM and PIA among the cool storage-time treatment groups in our study were likely due to storage-time periods and not from individual animal or testicle to testicle variability.

In this chapter under the Cool Storage section, the PPM and PIA reported for the domestic male goat when used as a model offers hope that postmortem epididymal sperm can be harvested from closely related endangered species for use in production of offspring using assisted reproductive technologies. The prefreeze PPM of the caprine sperm samples began a steady decline at 48 hours (55.7%) through to 96 hours of cool storage (41.9%). Even with this decrease, the PPM at 96 hours of cool storage was still maintained at a level that could be considered for use with standard caprine IVF procedures. These caprine epididymal sperm progressive motility values are greater than those reported by Anel et al.

(2000) for Spanish Cantabrian Chamois epididymal sperm collected during peak of the breeding season with progressive motility of 61.5% when harvested <120 hours postmortem.

There was an initial decrease in PIA from 24 hours (66.5%) to 48 hours (55.4%) and then PIA remained constant for the rest of the cool storage-time treatment groups to 96 hours (48.4%). The current prefreeze goat epididymal sperm PIA values were lower than the percent of live/dead sperm of 92% harvested from caprine bucks within 10 minutes postmortem and kept at 38°C by Blash et al. (2000). The goat epididymal sperm PIA values were comparable with the PPM values in the present study, indicating that these live, nonmotile sperm could be used to produce offspring with more advanced assisted reproductive technologies, such as with ICSI.

Two studies, Goto et al. (1990) and Goto (1993) reported the use of cryopreserved epididymal bovine sperm for the production of offspring with the aid of the ICSI procedure. Blastocyst development has also been achieved with ICSI using frozen-thawed caprine ejaculated sperm (Keskinetepe et al., 1997, 1998). The epididymal sperm collected by Blash et al. (2000) was cryopreserved and used for either AI or IVF. A live offspring was produced by AI and subsequently fertilization (40%) with embryos developing to the blastocyst stage (6%) was achieved with IVF using these epididymal sperm. Anel et al. (2000) indicated that this level of sperm quality from epididymal samples was at a minimum level good enough for sperm cryobanking, if used with specific assisted reproductive technologies.

In the Cryopreservation Experiment in Chapter 3, no significant differences were detected for the cryopreserved samples at 0 and 3 hours post-thaw for sperm maintained for 24, 48, 72 and 96 hours of cool storage at 4°C in the cauda epididymidis. Mean PPM

and PIA values for the caprine epididymal cryopreserved samples displayed an unusual upward trend from the 24-hour through to the 96-hour cool storage-time treatment groups at both 0 and 3 hours post-thaw.

This upward trend in both PPM and PIA for 0 and 3 hours post-thaw is not comparable to the values reported by this laboratory for the bovine male (Chapter 5). In bovine males, a trend was exhibited for a decreasing PPM and PIA from 24 to 96 hours of cool storage. Hishinuma et al. (2003) reported a slight increase in deer epididymal sperm viability after cool storage at 4°C from day 4 (80%) to day 7 (83%). This upward trend is similar to the upward trend noted for the caprine epididymal sperm that has been cryopreserved and then thawed. One possible explanation is a difference in the testicles allotted to the different cool storage-time treatment groups. This is not likely since none of the testicular parameters in this study could be correlated, either positively or negatively with the PPM or PIA values. Another possible explanation for this reversed trend is the simple attrition of weaker sperm cells with increased cool-storage time allowing more sperm to survive cryopreservation. The scientific literature has given no clear evidence to support this theory.

Cryopreserved caprine epididymal sperm that had been previously stored in the cauda epididymidis for 24 and 48 hours postmortem at 4°C exhibited an initial decrease in mean PPM values from prefreeze to 0 hour post-thaw. This same decrease in PPM from prefreeze to post-thaw, was noted for samples of deer epididymal sperm (Hishinuma et al., 2003) as well as samples of boar epididymal sperm (Kikuchi et al., 1998) that were cryopreserved after cool storage within the testicle for 0, 24 and 48 hours. This decrease in post-thaw PPM values for the 24- and the 48-hour cool storage-time treatment groups

might be explained by the lack of attrition of less viable sperm prior to cryopreservation, therefore, provoking higher rates of cryoinjury during the freeze-thaw process.

After 3 hours of co-incubation with Sperm TL medium, there was a decline in mean PPM values in the 24-hour cool storage-time treatment group from 0 hour to 3 hours post-thaw. This decrease from 0 to 3 hours post-thaw was indicative of greater sperm damage than initially occurred at 0 hour post-thaw. Ritar and Salamon (1982) reported the use of extended sperm culture for 6 hours post-thaw as a means of assessing the cryosurvivability of goat sperm. Latent cryodamage to sperm has also been reported by Saacke (1970), Fleming et al. (1976) and Chandler et al. (1978) for bull sperm with a decrease in both PPM and PIA values after incubating frozen-thawed samples for 3 hours at 37°C. A positive correlation was established in bull sperm for both PPM and PIA with fertility when assessed at 3 hours post-thaw. The 48-hour cool storage-time treatment group for the buck did not have a further decrease in mean PPM from 0 to 3 hours post-thaw in the present study.

Caprine epididymal sperm from both the 24- and the 48-hour cool storage-time treatment groups were similar over time for mean PIA values. Both the 24- and the 48-hour cool storage-time treatment groups initially had a decrease in PIA values from prefreeze to 0 hour post-thaw indicative of the sperm damage induced by cryopreservation. The decrease in PIA values for caprine epididymal sperm was similar to results reported by Hishinuma et al. (2003) for cryopreserved samples of deer epididymal sperm that had also been stored within the testis at 4°C for 24 and 48 hours.

In the present study, a large decrease (63%) was also detected between the prefreeze and the 3 hours post-thaw mean PIA values but no difference was noted between the sperm at 0 hour and 3 hours post-thaw. Blash et al. (2000) reported a decline in PIA

after thawing cryopreserved caprine epididymal sperm collected immediately postmortem. The mean PIA values reported were ~20% greater than the values from this study. Blash et al. (2000) subsequently used their frozen-thawed epididymal goat sperm for IVF and AI. Development to the blastocyst stage (6%) was noted for IVF and one pregnancy from 20 attempts was reported for AI with the cryopreserved epididymal sperm.

Epididymal sperm from the 72-hour cool storage-time treatment group did not show cryoinjury immediately at 0 hour post-thaw; however, a significant difference was detected between mean PPM at prefreeze and 3 hours post-thaw. The PIA data have the same pattern noted for the two previous cool storage-time treatment groups (24 and 48 hours). An initial decline in mean PIA occurred from prefreeze to 0 hour post-thaw but no further decline was noted, indicating that the sperm damage had occurred and that no evidence of cryoinjuries had occurred over the incubation period. Chauhan et al. (1994) reported a positive correlation between post-thaw sperm acrosome damage and the leakage of enzymes for Jamunapari goat males. The correlation of acrosomal damage to the release of enzymes can further be related to fertility because of the special role that the acrosome and its enzymes play in fertilization in mammalian species. Hishinuma et al. (2003) reported a decline in both PPM and PIA values for deer epididymal sperm that had been cryopreserved after cool storage within the testicles. Unlike the current study with goats, the study with deer had post-thaw differences immediately upon thawing and not after an extended post-thaw incubation period.

The 96-hour cool storage-time treatment group in the present study did not exhibit a difference in mean PPM values across any of the time measurements of prefreeze through 3 hours post-thaw. Even with no significant difference for mean PPM values in the 96-hour cool storage-time treatment group, the same trend was noted for mean PIA

values for the 24-, 48- and 72-hour cool storage-time treatment groups. Mean values for PIA in the 96-hour cool storage-time treatment group declined immediately from prefreeze to 0 hour post-thaw while the mean values between 0 hour and 3 hours post-thaw did not result in a significant difference.

Only the 96-hour cool storage-time treatment group exhibited the expected decline in PPM and PIA values from prefreeze through to 3 hours post-thaw in this study. The lack of a significant decrease in PPM values from prefreeze to 0 and 3 hours post-thaw was similar to that noted for the 96-hour cool storage-time treatment group of the stallion (Chapter 4) but different from the difference noted between prefreeze and 3 hours post-thaw in the 96-hour cool storage-time treatment group found in the bull (Chapter 5). The decrease in PIA for the 96-hour cool storage-time treatment for the goat is the same as that exhibited by both the stallion (Chapter 4) and the bull (Chapter 5).

Conclusion

Domestic caprine males used for these experiments demonstrated that sperm are hardy enough to withstand cool storage postmortem prior to harvest for potential use with assisted reproductive technologies. These findings can help to create an animal model beneficial to closely related exotic species. The decreasing trends detected for all four cool storage-time treatment groups in this study indicate that cryopreservation damages caprine epididymal sperm. Enough epididymal sperm motility and sperm membrane integrity remained after cooling in this experiment to warrant further research.

Song and Iritani (1988) and Blash et al. (2000) have used goat epididymal sperm for IVF and have achieved fertilization. Caprine offspring have also been produced with the use of epididymal sperm and AI (Blash et al., 2000). These cryopreserved sperm, even with cryodamage, could also be used to achieve fertilization and possibly blastocysts and

then offspring. These results indicate that sperm can be transported within the cauda epididymidis at 4°C for up to 96 hours postmortem, however, with some loss of progressive motility and/or percent intact acrosomes. Although there was success with the goat as well as other species in producing offspring with epididymal sperm, research is still needed to increase the success rate with the caprine species.

This study has also found that caprine epididymal sperm obtained from the cauda epididymidis stored at 4°C for 24, 48, 72 or 96 hours can undergo the freeze-thaw process and still produce motile and acrosome-intact sperm. As the time in cool storage increased the percent of progressive motile and/or viable sperm were reduced but with the use of IVF and ICSI even these low numbers of viable sperm in a sample could be used to produce embryos and possibly live offspring. Caprine offspring have been produced with the use of frozen-thawed ejaculated caprine sperm in this laboratory (Han et al., 2001).

If these findings can be applied toward exotic or endangered species that are closely related to domestic goats, then improvements can be made in the effort to preserve the genetically valuable gametes of deceased males in the wild or in captive settings. Further research needs to be continued with this domestic model to determine if there are better methods that can be used to cryopreserve epididymal sperm.

CHAPTER 4

PRESERVATION OF EQUINE EPIDIDYMAL SPERM STORED IN THE EPIDIDYMIDES OF TESTES AT 4°C FOR 24, 48, 72 AND 96 HOURS

Introduction

Interest in utilizing epididymal sperm from endangered species is high because of the inherent value of many individual males who die before making a contribution to the closed genetics of a captive population. Also, the ability to use epididymal sperm from males culled in wildlife parks could preserve their “wild” DNA for introduction into captive populations. The most efficient way to conserve the germplasm of endangered species would be to perfect the procedure using domestic animal models instead of experimenting on the more valuable samples of the endangered animals.

Whittingham (1968) reported on live mouse offspring after in vitro fertilization (IVF) with ejaculated sperm. Miyamoto and Chang (1972) reported on the development of IVF produced mouse embryos with cauda epididymal sperm. Fuller and Whittingham (1996) reported normal development to the fetal stage after IVF with mouse epididymal sperm that had been cooled to 4°C immediately post-collection. Studies have also reported fertilization with the use of rat epididymal sperm for IVF (Blandau and Rumery, 1961) as well as further embryo development and live offspring (Toyoda and Chang, 1974). Feline sperm collected from the ductus deferens has been reported for use in IVF and blastocyst production (Bowen, 1977) and from the cauda epididymidis for both IVF and intracytoplasmic sperm injection (ICSI) (Pushett et al., 2000).

Caprine epididymal sperm was also used to produce a live kid by the AI technique (Blash et al., 2000). Caprine epididymal sperm has also been reported for use in IVF production of embryos to the blastocyst stage (Song and Iritani, 1988; Blash et al., 2000).

Barker and Gandier (1957) also reported the birth of a foal produced by artificial insemination (AI) with frozen-thawed equine epididymal sperm. More recently, Morris et al. (2001) reported ongoing pregnancies in the domestic horse produced with frozen-thawed epididymal sperm introduced to the mare by hysteroscopic AI. A pregnancy was reported from AI with cryopreserved bovine epididymal sperm (Barker, 1954). Foote (2000) in a letter to the editor reported a live offspring by AI using bovine epididymal sperm. Previously, fertilization had been reported from IVF with bovine epididymal sperm (Ball et al., 1983). Graff et al. (1996) reported pregnancies with embryos produced by IVF using bovine epididymal sperm.

Bartels et al. (2001) reported the live birth of an eland produced by AI with frozen-thawed epididymal sperm. Meintjes et al. (1997) also reported the use of Burchell's zebra epididymal sperm for IVF with both Burchell's and Hartmann's zebra oocytes and development to the blastocyst stage.

Collection of viable epididymal sperm from various domestic species including feline (Pushett et al., 2000), canine (Stilley et al., 1999, 2000; Graff et al., 2000), caprine (Song and Iritani, 1988; Anel et al., 2000; Chapter 3), porcine (Kikuchi et al., 1998), equine (Bruemmer et al., 2002) and bovine (Foote, 2000) males have previously been described. Preliminary studies of harvesting epididymal sperm have also been reported with various laboratory species, such as mice (Kishikawa et al., 1999) and rats (Chapter 2), and exotic species including the African buffalo (Bezuidenhout et al., 1995; Friedmann et al., 2000), zebra (Bezuidenhout et al., 1995; Lubbe et al., 2000) and the Red hartebeest and eland (Bezuidenhout et al., 1995).

Most of these studies found that viable epididymal sperm could be harvested postmortem for potential use in the production of offspring using assisted reproductive

technologies. Each species demonstrated variation in the amount of time the sperm could remain within the cauda epididymidis prior to harvest, as well as, in the method of testes preservation resulting in the maximum number of viable sperm per animal. Most of these species have more viable sperm when testes were stored at 4 to 5°C prior to epididymal sperm collection when compared with testes storage at room temperature.

Unfortunately, with postmortem collections, samples most often need to be preserved for use in the future because the females are not available to produce the necessary oocytes for fertilization. Previous experiments have found that among domestic caprine males (Chapter 3), viable epididymal sperm can be collected from the cauda epididymides up to 96 hours postmortem when the testes are maintained at 4°C. To maintain the quality of postmortem-collected epididymal sperm, preservation methods need to be developed that can further increase the usability of the sperm over time. Blash et al. (2000) have reported that epididymal sperm collected immediately postmortem from the goat could be successfully cryopreserved. Epididymal sperm recovered <6 hours postmortem or postcastration from various African wild ruminants (Kilian et al., 2000), domestic stallions, zebra and rhinoceros (Lubbe et al., 2000), as well as, domestic dogs (Stilley et al., 2000) and cats (Pushett et al., 2000) can be preserved by cryopreservation with minimal loss in sperm motility. Stilley et al. (2000) also reported that canine epididymal sperm collected beyond 6 hours postmortem had enough survivability after cooling to make further research feasible.

Some of the endangered species that researchers have attempted to create assisted reproductive technologies to aid in species preservation are closely related to the domestic equine species. Therefore, the objective of this experiment was to determine the “window of opportunity” for collection of cauda epididymal sperm in an effort to create a research

model for exotic species that are most closely related to the domestic stallion. Also, to determine if the cryopreservation procedure used for preservation of ejaculated horse stallion sperm was applicable for preserving postmortem cauda epididymal sperm of equine males.

Materials and Methods (Cool Storage)

Experimental Design (Cool Storage)

Testicle pairs were obtained from mature stallions at slaughter and shipped cooled overnight to the Louisiana State University Embryo Biotechnology Laboratory at the beginning of the natural breeding season (May). Upon arrival, one testicle of each pair of testes was stored at 4°C for 48, 72 or 96 hours (n=6, 6 and 5 per storage-time treatment group, respectively) while the other testicle of a pair (control) was immediately processed (24-hour cool storage-time treatment group) (n=17). Percent progressively motile (PPM) and the percent of intact acrosomes (PIA) were evaluated by the same technician for each sample at each storage-time treatment period in this experiment.

Experimental Procedure (Cool Storage)

Equine testicles were collected as pairs from mature stallions at an abattoir in Dallas/Ft. Worth, TX. All testicle pairs were transported overnight individually packaged in Ziploc[®] bags, placed on ice at 4° C and shipped in a Styrofoam[™] shipping container by FedEx. The testicles were then processed in the following manner at each allotted treatment time period. Each testicle was allowed to warm to room temperature (25°C) from 4°C for up to 30 minutes. During this warming period each testicle was dissected away from the remaining tunica vaginalis.

Each testicle was positioned so that the corpus epididymidis was above the vas deferens. This placement allowed for the assessment of the side of origin of each testicle.

Measuring from the proximal (cranial) to the distal (caudal) pole and including the edge of the cauda epididymidis the testicular length was measured. A measurement was also taken midway between the ends of the caput and cauda epididymidis, excluding the unattached corpus segment to determine the circumference of each testicle.

Epididymal length was determined by following the external curvature with a string and referenced against a ruler. The testis plus the epididymis were weighed with a balance scale (American Family, Inc.). After weighing the testis and epididymis in its entirety, the epididymis was then carefully dissected away from the testis. Detached equine epididymides were weighed as one piece. The caudal section was separated and weighed before further processing.

Sperm TL medium (Parrish et al., 1988) was prepared by supplementing the original formulation with 0.006 g/ml of bovine serum albumin (Sigma, St. Louis, MO), 1mM sodium pyruvate and 25 µg/ml of gentamicin (GibcoBRL, Grand Island, NY). When completed, the medium was warmed in a water bath to 39°C for use with the harvested equine epididymal sperm.

The epididymis was minced and placed into a 60-mm plastic Petri dish (Becton Dickinson & Company, Franklin Lakes, NJ) after being weighed. This minced tissue was rinsed with Sperm TL medium (3 ml) warmed to 39°C. The epididymis was allowed to sit for <5 minutes to allow the sperm to be released from the equine cauda epididymidis. The tissue was removed and the epididymal sperm solution transferred to a 15-ml centrifuge tube (Corning, Inc., Corning, NY). The specimens were then centrifuged (Sorvall® Tabletop Refrigerated Centrifuge RT-6000, Sorvall® Centrifuges, Wilmington, DE) at 700 x g for 6 minutes to form an epididymal sperm pellet. The pellet was resuspended in Sperm TL medium and a 10-µl volume was removed from the suspension for analysis.

The cauda epididymidis sperm samples were assessed for PPM, PIA and a count of number of sperm obtained after processing. This was accomplished by removing a 5- μ l volume of sperm suspension and adding it to 95 μ l of Sperm TL medium for PPM and PIA analysis. The remaining 5- μ l epididymal sperm sample was added to 95 μ l of distilled water to create a 1:20 dilution for a hemacytometer count.

The PPM was assessed with a 10 μ l sample of the 1:20 sperm solution: Sperm TL medium dilution with a 100X objective and phase contrast microscopy (Nikon Labophot, Nikon Instruments, Inc., Lewisville, TX). Acrosomal integrity was assessed with an eosin B/fast green stained epididymal sperm specimen on a microscope slide (Aalseth and Saacke, 1986). Briefly, a 20- μ l volume of the 1:20 sperm solution was mixed with 10 μ l of eosin B/fast green stain and the two were co-incubated for 1 minute and that mixture was placed on a slide (10 μ l), spread over the surface and heat fixed. Each sample was examined by counting 200 sperm and placing sperm cells into two acrosome membrane categories. Sperm were categorized as having an intact acrosome if they retained membrane integrity and excluded the dye. Those sperm that appeared pink or red were considered membrane compromised and therefore not intact.

Statistical Analysis (Cool Storage)

Results of this study are expressed as a mean value \pm SEM for testicular and sperm parameters. Data were analyzed using a simple one-way ANOVA in SAS[®] (Version 8.0) with a Tukey multiple comparison adjustment (SAS, 1996). This analysis determined if there was a difference among 4°C cool storage-time treatment groups by examining the difference between PPM and/or PIA values found for each treatment group. The sperm PPM, PIA and epididymal sperm concentration were analyzed with the equine testicular

measurements (weight, length and circumference) as covariates to determine if differences occur among storage-time treatment groups were due to differences between individual stallion testes pairs.

Results (Cool Storage)

Testicular Parameters

Mean testicular weight, length, circumference, epididymides length, cauda epididymidis weight and number of sperm obtained per cauda epididymidis values for each storage-time treatment group are shown in Table 4.1. The mean total number of sperm collected per cauda epididymidis ranged from 2.7 ± 0.7 to $6.1 \pm 1.2 \times 10^9$ for all cool storage-time treatment groups. There was no significant difference found among any of the paired testicle measurements at the $P < 0.05$ level.

Parameters for Sperm Stored in Cauda Epididymidis at 4°C

Equine epididymal sperm mean PPM exhibited a significant decline ($P < 0.01$) from 24 and 48 hours to 72 and 96 hours (57.4 ± 3.3 , 41.7 ± 6.0 , 34.2 ± 5.5 and $32.0 \pm 3.7\%$, respectively) (Figure 4.1). Intact acrosomes from sperm stored in the cauda epididymides throughout the experiment are expressed as mean PIA values for 24, 48, 72 and 96 hours of cool storage-time treatment groups were 75.5 ± 2.2 , 71.4 ± 3.1 , 67.8 ± 4.5 and $65.0 \pm 3.4\%$, respectively. Also, a significant decline ($P < 0.01$) over time at 4°C in PPM was detected for 24- and 48-hour cool storage-time treatment groups compared with 72- and 96-hour cool storage-time treatment groups in this study. Sperm collected from the epididymis stored at 4°C retains adequate motility and maintains membrane integrity up to 96 hours postmortem.

Table 4.1. Mean (\pm SEM) testicular values from equine testes collected postmortem and stored at 4°C for 24, 48, 72 and 96 hours

Trt Group	Testicle Weight (kg)	Testicle Length (cm)	Testicle Circum.* (cm)	Epididymal Length (cm)	Cauda Weight (g)	No. Sperm [†] ($\times 10^9$)
24 h	0.231 \pm 0.01	11.8 \pm 0.3	17.2 \pm 0.4	25.5 \pm 1.0	7.75 \pm 0.58	5.6 \pm 1.0
48 h	0.184 \pm 0.02	11.2 \pm 0.4	16.3 \pm 0.7	25.0 \pm 0.7	6.95 \pm 0.87	2.7 \pm 0.7
72 h	0.217 \pm 0.02	12.0 \pm 0.8	16.9 \pm 1.2	26.9 \pm 1.9	7.37 \pm 0.73	4.2 \pm 1.2
96 h	0.245 \pm 0.03	12.6 \pm 0.8	17.7 \pm 1.0	25.8 \pm 1.4	8.58 \pm 0.91	6.1 \pm 1.2

*Circum. = circumference.

[†] No. Sperm = mean total number of sperm harvested per cauda epididymidis.
Means were not significantly different ($P > 0.05$).

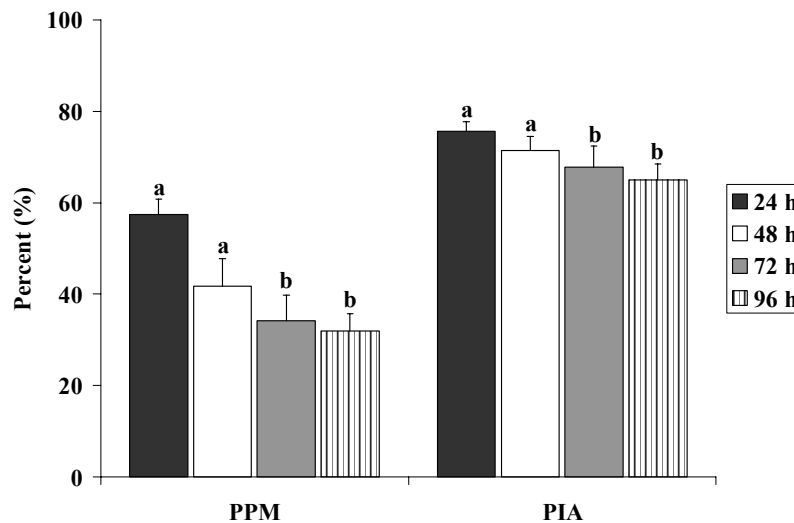


Figure 4.1. Mean (\pm SEM) percent progressive motility (PPM) and mean percent membrane integrity (PIA) for equine epididymal sperm stored in the cauda epididymidis of testes at 4°C for 24, 48, 72 or 96 hours (h). ^{a,b}Means with different superscripts within sperm parameters are significantly different ($P < 0.01$).

Materials and Methods (Cryopreservation)

Experimental Design (Cryopreservation)

Cryopreserved samples (15×10^6 progressively motile sperm/0.5 ml plastic straw) of equine epididymal sperm were collected during the Cool Storage experiment. Only testicular pairs from stallions that had 2 or more straws stored in liquid nitrogen (LN_2) for each storage-time treatment group were used for this experiment. Experimental samples included those that had been stored in the cauda epididymidis at 4°C for 24 hours ($n=15$), and 5 units each for the cool storage-time treatment groups of 48, 72 and 96 hours. Epididymal sperm were thawed and exposed to sperm analysis at prefreeze, 0 and 3 hours post-thaw. Each analysis included data from both PPM and PIA. An assessment was also made on the number of sperm that had a retained cytoplasmic droplet for prefreeze and 0 hour post-thaw.

Experimental Procedure (Cryopreservation)

Skim milk egg yolk with 4% glycerol (SM-EYG) extender (Varner et al., 1991) for equine sperm samples was made in one batch and frozen as 10-ml aliquots. On day of use, the SM-EYG extender was thawed at 4°C until liquidified and then warmed to 25°C .

During the Cool Storage Experiment in Chapter 4, when the initial epididymal sperm analysis (PPM, PIA and number of attached cytoplasmic droplets) had been completed the remaining sperm were prepared for cryopreservation. The equine epididymal sperm were centrifuged ($700 \times g$) to separate the sperm from the Sperm TL medium. The supernatant was removed from the epididymal sperm pellet and part A (0% glycerol) of the SM-EYG extender was added to create a 30×10^6 progressively motile sperm/ml dilution. The epididymal sperm samples were cooled to 4°C for a total of 2 hours. During this cooling period, part B (8% glycerol) of the SM-EYG extender was

added at 30-minute intervals to the sperm sample suspended in part A of the SM-EYG extender. Each 30-minute period added 0.125% of the total final volume. After equilibrating for 2 hours, the epididymal sperm sample was transferred into 0.5-ml plastic straws (Cassou straw, IMV Technologies, Minneapolis, MN) and placed into the freezing chamber.

Straws containing the diluted epididymal sperm sample were placed 2 cm above the level of the LN₂. The LN₂ vapor temperature at 2 cm was -110°C at the beginning of the freezing process. The temperature was monitored during the freezing process by a thermometer/thermocouple (Sensortek Model Bat-12, Costa Mesa, CA) that was inserted into the StyrofoamTM freezing chamber at the level of the straws. The straws were maintained at that level for 20 minutes until they reached -140°C and then they were immersed in LN₂. The rate of freezing from 4°C to -140°C was -7.2°C/minute for the epididymal sperm in this study.

All the straws of equine epididymal sperm were maintained in cryopreservation storage for a minimum of 24 hours prior to thawing for analysis. At thawing, sperm TL medium was prepared and warmed to 39°C. Straws of epididymal sperm were thawed in a 39°C water bath for 1 minute and then combined in a 15-ml centrifuge tube with 3 ml of pre-warmed Sperm TL medium. The epididymal sperm sample was then centrifuged twice at 700 x g for 6 minutes, supernatant removed and the pellet was resuspended with 2 ml of Sperm TL medium. Analysis consisted of the PPM and PIA completed at 0 and 3 hours post-thaw at 25°C. The number of attached cytoplasmic droplets was also assessed at 0 hour of post-thaw.

Statistical Analysis (Cryopreservation)

Progressively motile sperm, PIA and proximal cytoplasmic droplet retention are expressed as a mean value \pm SEM. Data were analyzed by a Proc Mixed protocol in SAS[®] (Version 8.0) (SAS, 1996) for a repeated measures design, with a Tukey multiple comparison adjustment. This analysis determined if there was a difference among cool storage-time treatment groups by comparing mean prefreeze and post-thaw values for PPM, PIA and cytoplasmic droplet parameters.

Results (Cryopreservation)

Epididymal Sperm PPM

Epididymal sperm mean PPM at prefreeze was not significantly different for cool storage-time treatment groups starting at 24 hours through to 96 hours (57.0 ± 3.3 , 41.7 ± 5.2 , 39.0 ± 5.7 and $35.0 \pm 6.3\%$, respectively) (Figure 4.2).

Epididymal sperm that had been cryopreserved had mean PPM values of 46.4 ± 2.4 , 38.4 ± 3.8 , 47.0 ± 4.2 and $42.5 \pm 4.7\%$ for 24, 48, 72 and 96 hours at 0 hour post-thaw, respectively. At 0 hour post-thaw there was no significant difference in PPM among the storage-time treatment groups.

The post-thaw analysis to identify latent cryodamage made after 3 hours of incubation resulted in mean PPM values for 24-, 48-, 72- and 96-hour cool storage-time treatment groups of 30.7 ± 3.3 , 23.4 ± 5.2 , 30.0 ± 5.7 and $28.8 \pm 6.4\%$, respectively. As with the prefreeze and the 0-hour post-thaw analyses, there was no significant difference for PPM among the storage-time treatment groups.

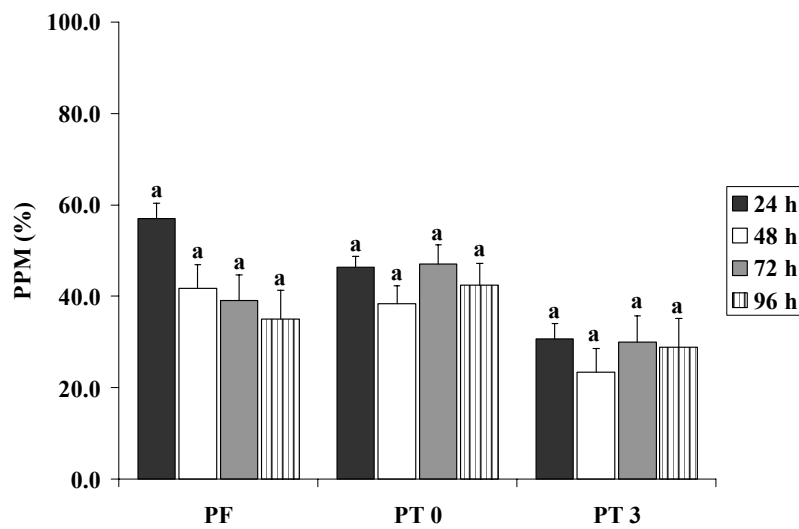


Figure 4.2. Mean (\pm SEM) percent progressive motility (PPM) at prefreeze (PF) and 0 and 3 hours post-thaw (PT) from cryopreserved equine epididymal sperm stored in the cauda epididymidis of testes at 4°C for 24, 48, 72 or 96 hours (h). These means were not significantly different ($P>0.05$).

Epididymal Sperm PIA

Epididymal sperm that maintained acrosomal membrane integrity were obtained from prefreeze samples stored throughout the experiment, and expressed as mean PIA of 76.7 ± 2.1 , 71.4 ± 3.3 , 71.4 ± 3.6 and $66.8 \pm 4.1\%$ for 24, 48, 72 and 96 hours, respectively (Figure 4.3). A similar downward trend as that noted for mean PPM values was detected for PIA among the cool storage-time treatment groups for prefreeze epididymal sperm.

The mean PIA values at 0 hour post-thaw for the 24-, 48-, 72- and 96-hour cool storage-time treatment groups were: 32.4 ± 3.0 , 34.2 ± 4.8 , 34.4 ± 5.2 and $17.5 \pm 4.4\%$, respectively. No significant difference was detected among the cool storage-time treatment groups for PIA at 0 hour post-thaw. As with the 0-hour sperm samples, the mean PIA values were less than PPM values at 3-hours post-thaw with values of 16.4 ± 2.3 , 21.4 ± 3.6 , 19.2 ± 3.9 and $35.0 \pm 4.4\%$ for 24, 48, 72 and 96 hours, respectively. Also, as with the prefreeze and the 0-hour post-thaw measurements, no difference was detected among the mean values for PIA for any of the cool storage-time treatment groups starting at 24 hours through 96 hours of cool storage at 4°C.

Cryopreserved samples, where the sperm had been stored in the testicle for 24, 48 and 72 hours, were significantly different ($P < 0.0001$) between the prefreeze and the 3 hours post-thaw mean PPM values as well as between 0-hour and 3-hours post-thaw values ($P < 0.0001$, $P = 0.0366$ and $P = 0.0249$ for 24, 48 and 72 hours, respectively) (Figure 4.4). However, the 96-hour cool storage-time treatment group did not exhibit a significant difference ($P > 0.05$) in PPM values for prefreeze, 0-hour and 3-hours post-thaw treatments.

There was a significant difference between prefreeze and 0-hour post-thaw mean PIA values for the cryopreserved sperm samples of all the cool storage-time treatment

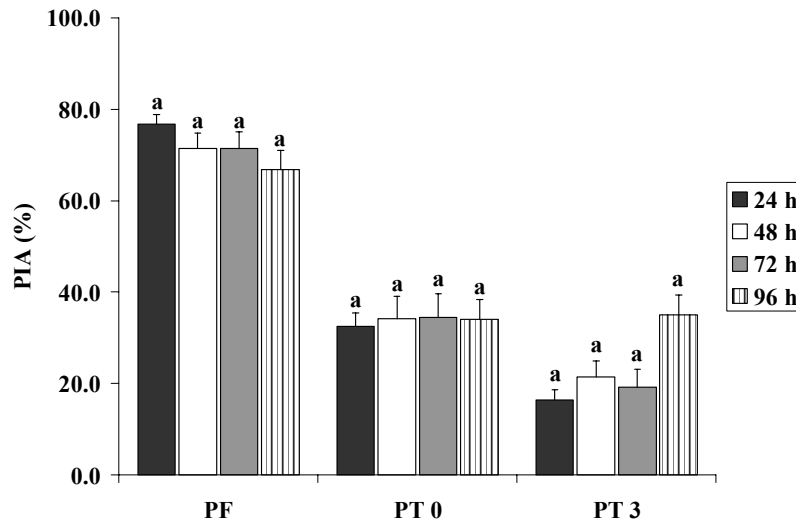


Figure 4.3. Mean (\pm SEM) percent sperm with intact acrosomes (PIA) at preefreeze (PF) and 0 and 3 hours post-thaw (PT) from cryopreserved equine epididymal sperm stored in cauda epididymidis of testes at 4°C for 24, 48, 72 or 96 hours (h). These means were not significantly different ($P > 0.05$).

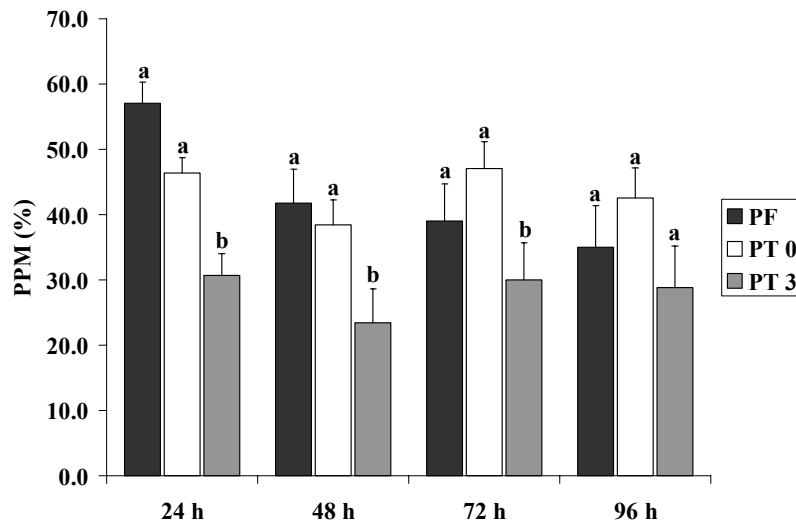


Figure 4.4. Mean (\pm SEM) percent progressive motility (PPM) from cryopreserved equine epididymal sperm stored in the cauda epididymidis of testes at 4°C for 24, 48, 72 or 96 hours (h). ^{a,b}Means with different superscripts within storage-time treatment groups are significantly different ($P < 0.0001$ for 24, 48 and 72 hours between preefreeze (PF) and post-thaw (PT) 3 h $P < 0.0001$ for 24 hour, $P = 0.0366$ for 48 hours and $P = 0.0249$ for 72 hours between post-thaw 0 h and post-thaw 3 h).

groups ($P<0.0001$ for 24 and 48 hours, $P=0.0005$ for 72 and 96 hours) as well as between prefreeze and 3 hours post-thaw ($P<0.0001$) (Figure 4.5). The 24-hour cool storage-time treatment group also had a significant difference ($P<0.0001$) between 0-hour and 3-hours post-thaw treatments for mean PIA values.

The number of epididymal sperm with an attached cytoplasmic droplet (CD) from each cryopreserved sample (15 for the 24-hour group and 5 for each of the 48-, 72- and 96-hour cool storage-time treatment groups) was counted using the eosin B/fast green stained slides from prefreeze and 0-hour post-thaw treatments. The mean percent of sperm with CD for prefreeze ($28.9\pm1.1\%$) was significantly greater ($P<0.0001$) than the mean for the 0-hour post-thaw treatment group ($7.8\pm1.0\%$). There was an inverse relation between the mean percent CD and the length of cool storage.

Discussion

The preliminary results from the mouse (Kishikawa et al., 1999; Stilley, 2002) the rat (Chapter 2) the dog (Stilley et al., 1999, 2000) and the goat male (Chapter 3) were found to have similar prefreeze sperm parameter patterns to those from this experiment. There was a gradual decline for prefreeze epididymal sperm parameters measured up to 96 hours of cool storage postmortem within the cauda epididymidis of the testicle stored at 4°C .

The mean testicular values described in these series of experiments were found to have a slight variation among the cool storage-time treatment groups (24 to 96 hours). However, when statistically analyzed as covariates for PPM and PIA, no significant differences were detected.

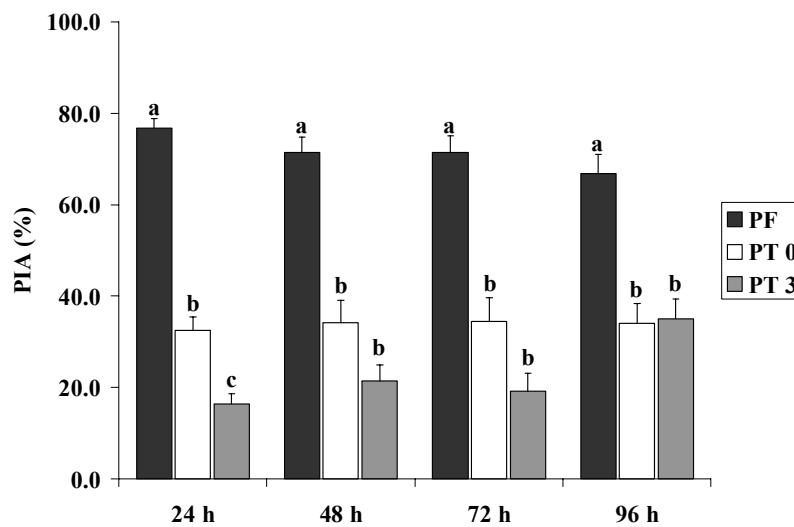


Figure 4.5. Mean (\pm SEM) percent sperm with intact acrosomes (PIA) from cryopreserved equine epididymal sperm stored in the cauda epididymidis of testes at 4°C for 24, 48, 72 or 96 hours (h). ^{a,b}Means with different superscripts between prefreeze (PF) and post-thaw (PT) 0 h within storage-time treatment groups are significantly different ($P < 0.0001$ for 24 and 48 hours, $P = 0.0005$ for 72 and 96 hours). ^{a,b}Means with different superscripts between prefreeze and post-thaw 3 h within storage-time treatment groups are significantly different ($P < 0.0001$). ^{a,c}Means with different superscripts between post-thaw 0 h and post-thaw 3 h within storage-time treatment groups are significantly different ($P < 0.0001$).

Percent progressively motile sperm assessed at 0 hour of post-thaw has been positively correlated with frozen bull sperm AI fertility in previous studies (Gibson and Graham, 1969; Linford et al., 1976; Kjaestad et al., 1993). The 3-hour analysis of PIA for frozen-thawed bull sperm has also been correlated with AI fertility (Chandler et al., 1978). No scientific literature has been found that positively or negatively correlates 0-hour post-thaw sperm parameters to fertility in the stallion.

Unlike the results reported for the human male (Patrizio et al., 1994), there was no correlation of epididymal length with fertility or other parameters used to estimate IVF fertility. Thus, the differences noted for the PPM and PIA among the cool storage-time treatment groups are due to the cool storage-time treatment periods or the cryopreservation and likely not individual animal variability.

There appears to be variation in the number of sperm harvested within the cool storage-time treatment groups. No significant difference ($P>0.05$) was noted among these mean values. The number of sperm collected does however appear to correlate to the weight of the cauda epididymis and the testicle. Varner et al. (1991) has reported a positive correlation between testicular volume and daily sperm production in the horse. The variation in harvested sperm numbers may, however, be due to the wide range of values within each group and the limited number of observations in some of the cool storage-time groups (e.g., 48, 72 and 96 hours of cool storage).

In the Cool Storage Experiment in Chapter 4, the domestic stallion epididymal sperm prefreeze PPM and PIA values showed that this method of storage in the cauda epididymidis at 4°C for an extended period of time (up to 96 hours of cool storage) produced favorable sperm viability results. The PPM and PIA values remained level from the 24-hour to the 48-hour treatment periods and then declined in the 72-hour treatment

period. The sperm values for PPM and PIA for the first 48-hour period remained level through the 72-hour to the 96-hour treatment periods. This pattern is similar to that reported by Lubbe et al. (2000) evaluating cooled epididymal sperm from two domestic horse stallions in South Africa. In the latter study, the PPM was maintained for greater than 132 hours when stored at 4°C within the cauda epididymidis of the testicle from these two stallions.

Equine epididymal sperm had greater PPM values in this study when compared with the range of 0 to 27% PPM for samples from nine stallions collected from the testis after 24 hours at 5°C (Bruemmer et al., 2002). The PPM values reported from the current study were greater than those from epididymal sperm of the Burchell's zebra reported by Bezuidenhout et al. (1995). The PPM of the zebra sperm samples exhibited a rapid decline from the time of death to 32 hours postmortem, with a final PPM of 10% at 32 hours. There was no indication of motility after storage within the cauda epididymidis for greater than 32 hours after death with the Burchell's zebra males.

Mean PPM patterns in the current study were also similar to and even slightly higher than the motility pattern reported by Torres-Boggino et al. (1995) for domestic horse stallion ejaculated semen stored for 72 hours at 5°C. The mean PPM pattern in our study ranged from 71% at 0 hour to 25% at 12 hours. With mean PPM values starting at 57.4% and ending with 32% this study again shows that this form of storage can be used postmortem to obtain sperm for germplasm banking. This approach could be useful for further studies for endangered species that are closely related to the horse by creating a model that would allow researchers to develop methodology with a domestic species for future use with endangered equid species.

In the Cryopreservation Experiment in Chapter 4, the number of testicles analyzed per cool storage-time treatment group decreased because of the loss of straws from some testicular pairs during the cryopreservation procedure. Analysis of the cauda epididymal sperm that survived the cryopreservation process did not produce any difference among 24-, 48-, 72- or 96-hour cool storage-time treatment groups at the prefreeze time measurement for either mean PPM or mean PIA values. This pattern was different from the results found in the Cool Storage Experiment in Chapter 4, where a decrease occurred in both PPM and PIA at 72 hours postmortem. There was a similar downward trend for prefreeze values noted in the Cool Storage Experiment in Chapter 4, however, the PPM and PIA values across time were not significantly different in the latter experiment.

There was no significant differences for the samples that had been cryopreserved and thawed for analysis at either 0 hour or 3 hours post-thaw when comparing 24-, 48-, 72- and 96-hour cool storage-time treatment groups. No differences among PPM or PIA values for cryopreserved samples were noted at this point with the caprine cauda epididymal sperm (Chapter 3). Mean PPM and PIA values for the equine epididymal cryopreserved samples did not display a downward trend from the 24-hour through to the 96-hour cool storage-time treatment groups but instead were found to have greater variability in their response to cryopreservation. Overall, sperm survival was the same for all cool storage-time treatment groups post-thaw.

Cryopreserved equine epididymal sperm that had been previously stored in the cauda epididymidis of the testicle for 24, 48 and 72 hours postmortem at 4°C did not display an initial decrease in mean PPM from prefreeze to 0 hour post-thaw. This initial response to the freeze-thaw process was also noted by Bruemmer et al. (2002), when stallion epididymal sperm was cryopreserved after 24 hours of storage within the testis at

5°C. Recently, Tiplady et al. (2002) reported a 66% decrease from prefreeze to post-thaw PPM for equine epididymal sperm cryopreserved immediately after collection. The decrease in PPM from prefreeze to post-thaw was similar to the response to cryopreservation in epididymal sperm from horse stallions reported by Lubbe et al. (2000). In the current study, after 3 hours of co-incubation with Sperm TL medium, mean PPM values decreased in the 24-hour through 72-hour cool storage treatment groups from 0 hour to 3 hours post-thaw. The decrease in PPM at 3 hours post-thaw is indicative of latent cryoinjury that was not found at 0 hour post-thaw.

In the current study, both the 72- and the 96-hour cool storage-time treatment groups exhibited an initial increase in PPM at 0 hour post-thaw before returning to the expected pattern at the 3-hour analysis. The prefreeze PPM was 40% and the PPM at 0 hour post-thaw was 50%. This initial increase in post-thaw PPM was also reported by Hopkins et al. (1988) after evaluating cryopreserved epididymal sperm of the gaur.

A possible explanation for the increase in PPM is that a greater loss of nonmotile sperm occurs during post-thaw processing than occurs in motile rich sperm fractions. This could explain the increase in motility noted in our study but it only occurred in two of the treatment groups and not all four of the groups. A second explanation could be that there was a large amount of motile but nonprogressive epididymal sperm in the prefreeze samples of these two groups (72- and 96-hours of cool storage-time treatment groups). It seems more likely due to the increased time of storage at a cool temperature that could have decreased the metabolism and therefore, the activity of a number of sperm for the prefreeze analysis of the 72- and 96-hour cool storage-time treatment groups.

The 24- through 72-hour cool storage-time treatment groups displayed similar downward differences over incremental analysis times for mean PIA values.

Cryopreserved equine epididymal sperm kept in cool storage in the testicle for 24, 48 and 72 hours initially showed a decrease from prefreeze to 0 hour post-thaw, indicative of the damage induced by the freeze-thaw process (Klug et al., 1992; Arav et al., 1994; Morris et al., 2000; Vidament et al., 2000). Others have reported damage to the sperm cell membrane are caused by the freeze-thaw process including an alteration of the sperm membrane components or the temperature phase transition that would make the membrane more prone to early fracture at stress points (Alvarez and Storey, 1992; Alvarez et al., 1993; Drobnis et al., 1993; Holt, 2000a). A difference was also detected between the prefreeze and the 3-hours post-thaw PIA values. The 24-hour cool storage-time treatment group also showed a decrease in PIA values between the 0-hour and 3-hours post-thaw time measurement, indicative of greater damage incurred by cryopreservation than initially assessed at 0-hour treatment. Post-thaw PIA values reported by Tiplady et al. (2002) for equine epididymal sperm cryopreserved immediately after collection had greater PIA values 47 to 50% than those found for the 24-hour to 96-hour cool storage periods of 34 to 17% in this experiment.

The 96-hour cool storage-time treatment group did not have a difference in mean PPM value across any of the time measurements of prefreeze through 3 hours post-thaw. Mean values for PIA in the 96-hour cool storage-time treatment group declined immediately from prefreeze to 0 hour post-thaw, while mean values between 0 hour and 3 hours post-thaw were not significantly different. The PIA trend noted for 24- through 72-hour treatment groups, had a similar pattern for mean PPM values in all four cool storage-time treatment groups. These patterns were found to have some similarities to those exhibited by the cryopreserved caprine cauda epididymal sperm subjected to the same storage-time treatment groups (Chapter 3).

The percentage of gaur epididymal sperm with a retained cytoplasmic droplet after freezing and then thawing was reported to decrease more than 50% in the thawed samples (Hopkins et al., 1988). In our study, the percentage of retained cytoplasmic droplets decreased from prefreeze (30%) to post-thaw (8%) for equine epididymal cryopreserved sperm. This loss of the cytoplasmic droplet was likely linked to the cryoinjury sustained by the sperm membrane. They attributed this loss to the osmotic changes associated with various factors, such as addition of glycerol, freezing or thawing. It has been reported that there is a higher incidence of cytoplasmic droplet retention for bull cauda epididymal sperm (Gupta et al., 1996) and for cat epididymal sperm (Axner et al., 1998) than that noted for ejaculated sperm. The presence of a retained cytoplasmic droplet does not appear to affect the results from IVF in the dog (Linde-Forsberg, 1995) and bull (Barth and Oko, 1989). However, Thundathil et al. (2001) reported that ejaculated bovine sperm with a higher percentage of cytoplasmic droplets used for IVF had a decrease in zona binding as well as arrested embryo development when oocytes did fertilize.

As noted in the cryopreserved caprine cauda epididymal sperm (Chapter 3), the downward trend in PPM and PIA found in all four cool storage-time treatment groups using equine cauda epididymal sperm indicates that cryopreservation does damage to the sperm, however, the damage is less for the equine sperm than for those of the caprine species. Barker and Gandier (1957) have produced a live foal using frozen-thawed equine epididymal sperm by AI. More recently, Morris et al. (2001) also has reported pregnancies from mares inseminated with frozen-thawed epididymal sperm by hysteroscopic AI.

Using these cryopreserved samples to create embryos using current assisted reproductive technologies would be difficult for the equids at this time. At present, the steps have not been worked out that would make IVF or ICSI efficient as a means of

preserving a genetic line with frozen-thawed epididymal sperm. Palmer et al. (1991) reported the live birth of a filly (female foal) after IVF with fresh stallion sperm. Fertilization (0 to 79%) and embryo development to the blastocyst stage (18.2%) was reported for in vitro matured oocytes subjected to fresh ejaculated stallion sperm by zona drilling (Li et al., 1995). In recent attempts, fertilization has been achieved with frozen-thawed stallion sperm using IVF but embryo development was not reported (Alm et al., 2001). Cochran et al. (1998, 2000) has produced equine offspring using ICSI with ejaculated stallion sperm. Fertilization of 90 to 93% was achieved without chemical activation of sperm and with fresh and frozen-thawed equine sperm by ICSI (Choi et al., 2001). When these procedures have been improved then genetically valuable epididymal sperm could be used to produce embryos and subsequently live offspring.

Conclusion

The domestic horse stallions used for these experiments to determine the status of sperm stored in the cauda epididymidis of the testicle at 4°C for 24 to 96 hours, showed that sperm are hardy enough to withstand postmortem cool storage prior to harvest as well as produce epididymal sperm that can undergo the freeze-thaw process and still produce progressively motile, plasma membrane-intact sperm. Findings from the domestic horse stallion have helped to create a model that could be beneficial to closely related exotic equids. Instead of wasting valuable genetics to determine the correct “window of opportunity” or methods to be used for sperm banking, this domestic species could be used to establish basic parameters.

These experimental results indicate that sperm can be stored within the cauda epididymidis of the testicle at 4°C for up to 96 hours postmortem with only minor losses in motility and acrosomal integrity. Motile and/or acrosome-intact sperm may be low in a

sample for standard AI procedures; however, with the use of IVF and ICSI a low number of sperm could be used to produce embryos and subsequently live offspring. Further research needs to be continued with these domestic models to determine if there are better methods or materials that can be used to preserve epididymal sperm.

Scientists have already been investigating the use of epididymal sperm for the production of offspring using assisted reproductive technologies. Although there has been some success with both the equine and other species in producing offspring, much work is still needed to produce greater success with the domestic horse.

CHAPTER 5

PRESERVATION OF BOVINE EPIDIDYMAL SPERM STORED IN THE EPIDIDYMIDES OF TESTES AT 4°C FOR 24, 48, 72 AND 96 HOURS

Introduction

Many recent experiments have evaluated the efficacy of postmortem epididymal sperm collection to aid in the preservation of valuable genetic material. It was determined that sperm can be harvested postmortem with a minimal amount of loss of motility for several different domestic species including: mice (An et al., 1999b; Stilley, 2002), rats (Chapter 2), dogs (Stilley et al., 1999, 2000), goats (Anel et al., 2000; Chapter 3), horses (Lubbe et al., 2000; James et al., 2002) and cattle (Foote, 2000). Harvesting the sperm from the cauda epididymidis can also be delayed for a number of days if either the deceased animal or the testicles are stored at 4 to 5°C (An et al., 1999b; Anel et al., 2000; Friedmann et al., 2000; Lubbe et al., 2000; Stilley et al., 2000; Chapters 2, 3 and 4). Harvesting viable sperm is one of many important steps toward creating successful assisted reproductive technologies aimed at saving endangered species.

Iwamatsu and Chang (1971) have reported successful fertilization of mouse oocytes using epididymal sperm and Miyamoto and Chang (1972) reported on the further development of in vitro fertilization (IVF) produced mouse embryos with epididymal sperm. Fuller and Whittingham (1996) reported the production of normal mouse fetuses after IVF with epididymal sperm that had been cooled to 4°C immediately after collection. Studies have also been reported describing the use of rat epididymal sperm for IVF (Blandau and Rumery, 1961) and further embryo development and production of live offspring (Toyoda and Chang, 1974; Songsasen et al., 1997).

Success has also been reported in the rabbit with IVF and embryo development to the blastocyst stage using fresh epididymal sperm (Ogawa et al., 1972; Hosoi et al., 1981). Feline sperm collected from the ductus deferens has been used in IVF and blastocyst production (Bowen, 1977). More recently, fresh sperm from the cauda epididymidis resulted in fertilization achieved by IVF (Niwa et al., 1985), with blastocyst production by IVF (Pushett et al., 2000) and by intracytoplasmic sperm injection (ICSI) with fresh and frozen-thawed sperm (Pushett et al., 2000; Bogliolo et al., 2001).

Caprine frozen-thawed epididymal sperm was used to produce one live kid using AI (Blash et al., 2000). Preserved (18 hours at 20° to 25°C) goat epididymal sperm has also been used for the IVF production of embryos developing to the blastocyst stage (Song and Iritani, 1988; Blash et al., 2000). Porcine epididymal sperm stored in the epididymides at 4°C for up to 3 days postmortem and then cryopreserved was found to be capable of fertilization using IVF procedures (Kikuchi et al., 1998). Barker and Gandier (1957) also reported the birth of a foal produced by artificial insemination (AI) with frozen-thawed equine epididymal sperm.

A pregnancy was reported by Barker (1954) from AI with frozen-thawed bovine epididymal sperm. Foote (2000) reported the production of a calf by AI using cooled (5°C) bovine epididymal sperm. Graff et al. (1996) reported pregnancies from IVF-derived embryos using bovine epididymal sperm. Also, the production of live calves was reported after the transfer of bovine embryos created by using the ICSI technique with frozen-thawed epididymal sperm (Goto et al., 1990; Goto, 1993).

Other groups have reported the use of epididymal sperm for IVF in exotic species, such as the lion (Jewgenow et al., 1997; Bartels et al., 2000), puma (Jewgenow et al., 1997), blesbok (Winger et al., 1997) and African buffalo (Shaw et al., 1995). Bartels et al.

(2001) reported the production of a live offspring from an eland with AI using frozen-thawed epididymal sperm. Meintjes et al. (1997) also reported the use of Burchell's zebra epididymal sperm for IVF with both Burchell's and Hartmann's zebra oocytes and with subsequent development to blastocysts. A pregnancy was also reported by Hopkins et al. (1988) in a domestic cow inseminated with frozen-thawed gaur epididymal sperm.

Numerous experiments have attempted to determine the time constraints for collecting viable epididymal sperm from domestic species including feline (Pushett et al., 2000), canine (Stilley et al., 1999, 2000; Graff et al., 2000; Yu and Leibo, 2002), porcine (Kikuchi et al., 1998), caprine (Song and Iritani, 1988; Anel et al., 2000; Chapter 3), equine (Bruemmer et al., 2002; James et al., 2002) and bovine (Igboeli and Foote, 1968; Foote, 2000) males. Preliminary studies on postmortem epididymal sperm collection have also been completed with various laboratory species including the mouse and the rat (Kishikawa et al., 1999; Stilley, 2002; Chapter 2), and exotic species including the African buffalo (Bezuidenhout et al., 1995; Friedmann et al., 2000), zebra (Bezuidenhout et al., 1995; Lubbe et al., 2000), the Red hartebeest and eland (Bezuidenhout et al., 1995). It was reported that greater numbers of viable epididymal sperm were maintained when the testes were stored at 4 to 5°C prior to sperm collection. These studies found that viable epididymal sperm could be harvested postmortem for possible use in assisted reproductive technologies.

A number of the endangered species are currently being studied to develop cryopreservation procedures are closely related to domestic cattle. Therefore, the objective of the Cool Storage Experiment in Chapter 5 was to determine the “window of opportunity” for collection of epididymal sperm from the domestic bull to create a research model for closely related exotic species.

Previous experiments in this laboratory have found that domestic caprine and equine males have viable epididymal sperm up to 96 hours postmortem when the intact testes are maintained at 4°C (James et al., 2002; Chapter 3). To increase the value of postmortem-collected epididymal sperm, preservation methods need to be developed that further increase the utility of the harvested sperm over time.

Blash et al. (2000) reported that epididymal sperm collected immediately postmortem from the goat can be successfully cryopreserved. Kikuchi et al. (1998) successfully cryopreserved porcine epididymal sperm harvested after storage at 4°C in the epididymides for up to 3 days postmortem. Epididymal sperm recovered post-castration or <6 hours postmortem, from several African wild ruminants (Kilian et al., 2000), domestic stallions, zebras and a rhinoceros (Lubbe et al., 2000), as well as with dogs (Stilley et al., 2000) and cats (Pushett et al., 2000), can be cryopreserved with minimal loss of motility. Hopkins et al. (1988) collected cauda epididymal sperm at 27 hours postmortem from gaur bulls and subsequently cryopreserved the epididymal sperm for AI. Stilley et al. (2000) also found that canine epididymal sperm collected beyond 6 hours postmortem displayed enough survivability after cryopreservation to stimulate further research. The use of cryopreserved epididymal sperm for IVF in exotic species has been reported in animals, such as the lion and puma (Jewgenow et al., 1997) and blesbok (Winger et al., 1997).

The purpose of the Cryopreservation Experiment in Chapter 5 was to determine if a cryopreservation technique utilized for preservation of ejaculated sperm from domestic bulls was effective for preserving postmortem epididymal samples so that these samples could be used later with assisted reproductive technologies to produce live offspring.

After determining the “window of opportunity” for postmortem collection as well as confirming the ability to store the epididymal sperm on a long-term basis, the logical

next step would be to determine the efficacy of IVF for the production of embryos and offspring. Several studies have started with the most basic assisted reproductive technique, such as AI, and have produced live offspring (Barker, 1954; Barker and Gandier, 1957; Bartels et al., 2001). In all these cases, the sperm were harvested from the epididymis directly postmortem and processed for cryopreservation. Success has also been reported on the production of live mouse pups using IVF and ICSI with epididymal sperm harvested as late as 20 days postmortem (Kishikawa et al., 1999).

The first live calf born from IVF with ejaculated sperm was reported by Brackett et al. (1982), however, no scientific literature has reported the production of a live calf using IVF with bovine epididymal sperm. Goto et al. (1990) and Goto (1993) also reported two live births when they used frozen-thawed bovine epididymal sperm in conjunction with ICSI. Several studies have found that epididymal sperm can be used to create embryos and/or offspring with assisted reproductive technologies (Brackett et al., 1978; Song and Iritani, 1988; Goto et al., 1990; Goto, 1993; Graff et al., 1996). The objective of the IVF Experiment in Chapter 5 was to determine the efficacy of IVF, used in conjunction with cryopreserved epididymal sperm harvested at 24 and 48 hours postmortem to produce embryos.

Materials and Methods (Cool Storage)

Experimental Design (Cool Storage)

Testicular pairs were collected from mature beef-type bulls after slaughter and shipped overnight by FedEx in a StyrofoamTM container at 4°C as pairs in plastic bags. Upon arrival, one of the testicles was stored at 4° C for 48, 72 or 96 hours (n=10 for each storage-time treatment group) while the other testicle (control) of a pair was immediately processed (24-hour cool storage-time treatment group) (n=30). Percent progressively

motile (PPM) and percent intact acrosomes (PIA) were evaluated by the same technician for each sample at each storage-time treatment period of the experiment.

Experimental Procedure (Cool Storage)

Bovine testicles were collected as pairs from mature beef-type bulls at an abattoir in Dallas/Ft. Worth, TX. All testicle pairs were transported overnight to the Louisiana State University Embryo Biotechnology Laboratory individually packaged in Ziploc[®] bags, placed on ice at 4° C and shipped in a Styrofoam[™] igloo container by FedEx.

Each testicle was allotted to its respective treatment time group and processed. After removal from storage (4°C), each testicle was allowed to warm to room temperature (25°C) for not more than 30 minutes prior to processing. During this warming period, the testicle was dissected away from its tunica vaginalis and other extraneous tissue.

Each testicle was positioned so that the corpus epididymidis was on top of the vas deferens. This placement allowed for the assessment of the side of origin of each testicle. Testicular length was measured from proximal (cranial) to distal (caudal) pole, including the edge of the cauda epididymidis. The circumference of each testicle was measured midway between the ends of the caput and cauda epididymidis. For the bovine testis in this study, this included the attached corpus epididymidis.

Epididymal length was determined by following the external curvature with a string that was then referenced against a ruler. The weight of the testis plus the epididymis was then recorded using a balance scale (American Family, Inc.). The epididymis was then carefully dissected away from the testis. The epididymis was sectioned and weighed as caput, corpus and cauda using a Mettler Toledo metric scale (Model #AE 50, Columbus, OH).

Sperm TL medium (Parrish et al., 1988) was prepared with the addition of 0.006 g/ml of bovine serum albumin (Sigma, St. Louis, MO), 1mM sodium pyruvate and 25 µg/ml of gentamicin (GibcoBRL, Grand Island, NY) and warmed in a water bath to 39°C prior to use with the epididymal sperm.

Upon dissection from the testis, the epididymis was minced and placed into a 60-mm plastic Petri dish (Becton Dickinson & Company, Franklin Lakes, NJ). The tissue was rinsed with 3 ml of Sperm TL medium previously warmed to 39°C and allowed to sit for 5 minutes. After 5 minutes, the tissue was removed to a 15-ml centrifuge tube (Corning, Inc., Corning, NY) and then centrifuged (Sorvall® Tabletop Refrigerated Centrifuge RT-6000, Sorvall® Centrifuges, Wilmington, DE) at 700 x g for 6 minutes to create a sperm pellet. The supernatant was discarded and the pellet was resuspended in Sperm TL medium to create a 300×10^6 sperm/ml suspension. A 10-µl volume was removed from the suspension for analysis.

Sperm analysis included PPM, PIA and a count of number of sperm obtained from the epididymis. A 5-µl volume of sperm suspension was added to 95 µl of Sperm TL medium for PPM and PIA analysis. The remaining 5 µl was added to 95 µl of distilled water to create a 1:20 dilution for a hemacytometer count. Motility was assessed on 10 µl of 1:20 sperm solution:Sperm TL medium dilution with a Nikon Labophot 100X phase contrast microscope (Nikon Instruments, Inc., Lewisville, TX). An eosin B/fast green stained epididymal sperm specimen on a microscope slide was used to assess PIA. A 20-µl volume of the 1:20 sperm solution was mixed with 10 µl of eosin B/fast green stain (Aalseth and Saacke, 1986). After co-incubating for 1 minute, the mixture was placed on a slide (10 µl) and spread over the surface and quickly heat fixed. Each sample was

analyzed by counting 200 sperm and respective category determined. Exclusion of the dye indicated an intact cellular membrane and a viable cell. Those sperm that appeared pink or red were considered membrane compromised and therefore nonviable.

Statistical Analysis (Cool Storage)

Bovine testicular parameters (e.g., testicular weight, length and circumference and epididymal weight and length) and epididymal sperm parameters (e.g., motility and viability) are expressed as a mean value \pm SEM. Testicular parameters and sperm values were analyzed using a simple one-way ANOVA in SAS[®] (Version 8.0) with a Tukey multiple comparison adjustment (SAS, 1985). The epididymal sperm parameters were analyzed with testicular measurements as covariates to determine if differences detected among storage-time treatment groups were due to differences among individual bull testicles or due to the amount of time that testicles were kept at 4°C prior to sperm collection.

Results (Cool Storage)

Testicular Parameters

Bovine mean testicular values for testicular weight, length, circumference, epididymal length, caput epididymidis weight, corpus epididymidis weight, cauda epididymidis weight and total number of sperm obtained from each cool storage-time treatment group are displayed (Table 5.1). There were no significant differences detected ($P>0.05$) among any of the paired testicle measurements. There was also no difference found among the mean total number of sperm collected from the cauda epididymis in each cool storage-time treatment group with values ranging from 323 ± 85 to $558\pm238 \times 10^6$ sperm.

Table 5.1. Mean (\pm SEM) testicular values from bovine testes collected postmortem and stored at 4°C for 24, 48, 72 and 96 hours

Trt Group	Testicle Weight (kg)	Testicle Length (cm)	Testicle Circum.* (cm)	Epididymal Length (cm)	Cauda Weight (g)	No. Sperm [†] ($\times 10^6$)
24 h	0.383 \pm 0.02	19.0 \pm 0.3	20.9 \pm 0.4	34.1 \pm 0.6	8.8 \pm 0.5	405 \pm 86
48 h	0.389 \pm 0.02	18.7 \pm 0.7	20.5 \pm 0.5	34.5 \pm 0.8	8.4 \pm 0.6	558 \pm 238
72 h	0.405 \pm 0.02	19.3 \pm 0.5	22.1 \pm 0.5	34.7 \pm 1.1	9.3 \pm 0.6	334 \pm 65
96 h	0.353 \pm 0.04	19.0 \pm 0.6	20.2 \pm 0.6	33.1 \pm 1.2	8.7 \pm 1.5	323 \pm 85

*Circum. = circumference.

[†] No. Sperm = mean total number of sperm harvested per cauda epididymidis.

Means were not significantly different ($P > 0.05$).

Parameters for Sperm Stored in the Cauda Epididymidis at 4°C

The PPM values for bovine epididymal sperm had a significant decline ($P < 0.01$) from 24 to 48 hours and again from 72 to 96 hours in the cool storage-time treatment groups (64.8 \pm 2.2, 49.0 \pm 4.4, 46.0 \pm 4.5 and 30.0 \pm 3.9%, respectively) (Figure 5.1). Plasma membrane intact sperm were obtained from sperm stored throughout the experiment, with an initial decrease occurring between 24 and 48 hours and then maintaining mean PIA values from 48 through 96 hours of the cool storage-time treatment groups (76.9 \pm 1.5, 64.9 \pm 4.7, 52.9 \pm 4.2 and 53.5 \pm 2.8%, respectively). Bovine epididymal sperm collected from the cauda epididymidis stored at 4°C retains adequate motility as late as 72 hours postmortem and gives all indications of maintaining sperm membrane integrity up to 96 hours postmortem.

Materials and Methods (Cryopreservation)

Experimental Design (Cryopreservation)

Cryopreserved samples of bovine cauda epididymal sperm (15×10^6 progressive motile sperm/0.5 ml straws) were collected during the Cool Storage Experiment in Chapter

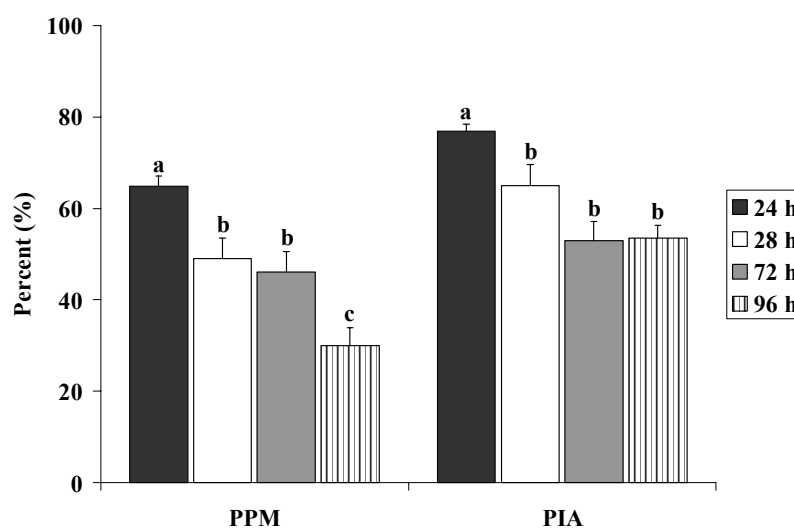


Figure 5.1. Mean (\pm SEM) percent progressive motility (PPM) and mean percent membrane integrity (PIA) for bovine epididymal sperm stored in the testicle at 4°C for 24, 48, 72 or 96 hours (h). ^{a,b,c}Means with different superscripts within sperm parameters are significantly different ($P<0.01$).

5. Bull testicular pairs with 2 or more straws in liquid nitrogen (LN₂) were included in this experiment (n=26, 8, 9 and 9 for 24-, 48-, 72- and 96-hour cool storage-time treatment groups, respectively). Epididymal sperm were thawed and exposed to sperm analysis at 0 and 3 hours post-thaw. Each analysis included PPM and PIA. Analysis at prefreeze and 0 hour post-thaw included a count of the number of epididymal sperm with a retained cytoplasmic droplet.

Experimental Procedure (Cryopreservation)

For medium preparation, 0.006 g/ml of bovine serum albumin (Sigma, St. Louis, MO), 1mM sodium pyruvate and 25 µg/ml of gentamicin (GibcoBRL, Grand Island, NY) were added to Sperm TL medium (Parrish et al., 1988). When the medium was completed, it was warmed in a water bath to 39°C prior to its use with the epididymal sperm. The cryoprotective medium utilized for preservation of bovine cauda epididymal sperm was egg-yolk citrate with 7% glycerol (EYG) (Salisbury et al., 1941). This medium was previously prepared as one batch and frozen for subsequent use in 10-ml aliquots.

When the number of sperm per cauda epididymidis and PPM had been determined on the samples during the Cool Storage Experiment in Chapter 5, the remainder of the sperm sample was then prepared for cryopreservation. Each sperm sample was centrifuged for 6 minutes at 700 x g to obtain separation of sperm from Sperm TL medium. The supernatant and the epididymal sperm pellet was resuspended in part A (0% glycerol) of EYG extender to create a 30×10^6 sperm/ml dilution. This epididymal sperm dilution was cooled at 4°C for a total of 1 hour while additions of part B (14% glycerol) EYG were made at 15-minute increments. Each addition of part B EYG was 0.125% of the total final volume to be cryopreserved. After 1 hour, the bovine epididymal sperm solution was aspirated into 0.5-ml plastic straws (Cassou straw, IMV Technologies, Minneapolis, MN)

and placed into the StyrofoamTM freezing chamber. The freezing chamber was a StyrofoamTM cooler containing a metal rack to hold the cryostraws 2 cm above the LN₂. A thermosensor (Sensortek Model Bat-12, Costa Mesa, CA) was inserted into the StyrofoamTM cooler 2 cm above the LN₂ to record the temperature change during the cryopreservation process.

The straws were placed 2 cm above the level of the LN₂. The starting temperature at 2 cm was -110°C at the beginning of the cryopreservation process. The freezing protocol for the bovine cauda epididymal sperm required that the straws remain at this level for 8 minutes until they reached -100°C at which time they were rapidly frozen by immersion into the LN₂ (-196°C). The rate of freezing was -13°C/minute for the epididymal sperm going from 4°C to -100°C.

Epididymal sperm were cryopreserved for at least 24 hours prior to thawing for analysis. On day of post-thaw sperm analysis, Sperm TL medium was prepared and warmed to 39°C. Straws of cryopreserved epididymal sperm were removed from LN₂ and placed into a 39°C water bath for 1 minute. The samples were then combined in a 15-ml centrifuge tube with 3 ml of pre-warmed Sperm TL medium. The sperm mixture was centrifuged at 700 x g for 6 minutes and the supernatant removed from the sperm pellet. This pellet was then resuspended with another 3 ml of Sperm TL medium and washed once more. Sperm pellets were resuspended in 2 ml of Sperm TL medium and prepared for analysis.

Analysis consisted of the PPM, PIA and number of retained cytoplasmic droplets evaluated at room temperature (25°C) at 0 hours post-thaw as well as PPM and PIA at 3 hours post-thaw. Percent progressively motile sperm assessed at 0 hour of post-thaw has been reported to positively correlate with frozen-thawed sperm fertility of bulls (Gibson

and Graham, 1969; Linford et al., 1976; Kjaestad et al., 1993). The 3-hour analyses of PPM, as well as PIA, have also been positively correlated with frozen-thawed sperm fertility of bulls (Saacke, 1970; Fleming et al., 1976; Chandler et al., 1978). PIA has also been positively correlated with measurable enzyme leakage in frozen-thawed goat sperm (Chauhan et al., 1994) and subsequent fertilization.

Statistical Analysis (Cryopreservation)

The sperm parameters (PPM, PIA and number of retained cytoplasmic droplets) are expressed as a mean value \pm SEM per treatment group. Data were analyzed using Proc Mixed in SAS[®] (Version 8.0) (SAS, 1996) and a repeated measures design, with a Tukey multiple comparison adjustment. This analysis determined if there was a difference among cool storage-time treatment groups (24, 48, 72 and 96 hours) by examining the difference between the prefreeze and post-thaw PPM and prefreeze and post-thaw PIA bovine epididymal sperm values.

Results (Cryopreservation)

Epididymal Sperm PPM

The mean prefreeze PPM values for 24-, 48-, 72- and 96-hour cool storage-time treatment groups were 64.4 ± 2.6 , 49.0 ± 4.2 , 47.8 ± 4.5 and $32.8 \pm 5.1\%$, respectively (Figure 5.2). The prefreeze PPM for epididymal sperm had a significant decline ($P < 0.0001$) between 24 and 96 hours of cool storage-time treatment groups.

Mean PPM values at 0 hour post-thaw were 24.8 ± 3.5 , 23.0 ± 5.6 , 20.0 ± 5.9 and $11.4 \pm 6.7\%$ for 24-, 48-, 72- and 96-hour cool storage-time treatment groups, respectively. The post-thaw analysis made after 3 hours of incubation at 37°C had mean PPM values for 24-, 48-, 72- and 96-hour cool storage-time treatment groups of 16.2 ± 2.9 , 15.0 ± 4.7 ,

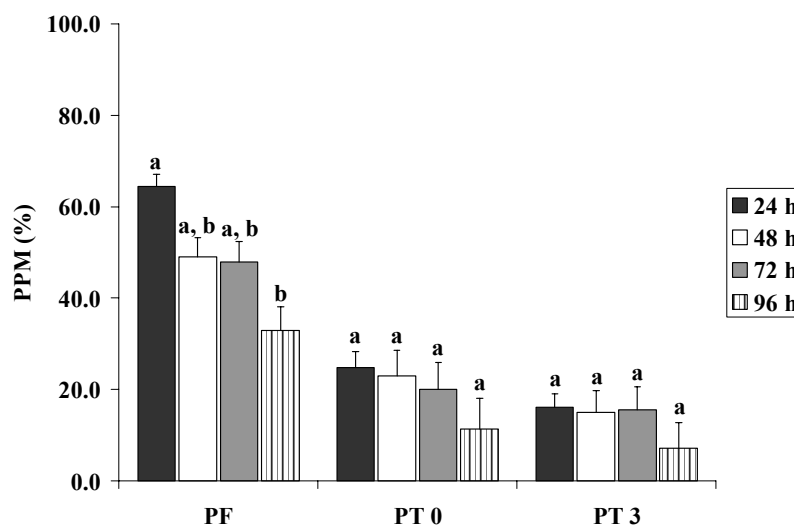


Figure 5.2. Mean (\pm SEM) percent progressive motility (PPM) at prefreeze (PF) and 0 and 3 hours post-thaw (PT) from cryopreserved bovine epididymal sperm stored in the cauda epididymidis of testes at 4°C for 24, 48, 72 or 96 hours (h). ^{a,b}Means with different superscripts within time of measurement are significantly different ($P < 0.0001$).

15.6±5.0 and 7.1±5.6%, respectively. No significant difference was detected among the mean values for PPM for any of the cool storage-time treatment groups at either the 0-hour or 3-hour post-thaw incubation.

Epididymal Sperm PIA

Sperm with intact acrosomal membranes were obtained from prefreeze samples stored throughout the experiment, with mean PIA values for 24, 48, 72 and 96 hours of the cool storage-time treatments were 76.9±2.1, 64.9±3.4, 54.1±3.6 and 52.3±5.0%, respectively (Figure 5.3). Significant differences were noted between the 24-hour and 72-hour cool storage-time treatment groups, as well as, between the 24-hour and 96-hour cool storage-time treatment groups ($P \leq 0.0001$).

The mean PIA values at 0 hour post-thaw for the 24-, 48-, 72- and 96-hour cool storage-time treatment groups were 27.7±2.6, 23.6±4.2, 25.2±4.5 and 27.2±5.1%, respectively. Mean PIA values remained larger than the mean PPM values at 3 hours post-thaw with values of 20.1±2.6, 13.6±4.2, 16.3±4.4 and 22.6±5.0% for 24, 48, 72 and 96 hours of cool storage-time treatments, respectively. No difference was detected among the four cool storage-time treatment groups for PIA at 0 hour or 3 hours post-thaw.

Significant differences were detected from mean prefreeze to post-thaw analysis in all of the cool storage-time treatment groups are presented in Figure 5.4. Cryopreserved sperm samples that had been stored in the testicle for 48 and 72 hours of the cool storage-time treatments were found to have a significant difference ($P=0.0021$ and 0.0026 , respectively) between mean prefreeze (49.0±4.2% and 47.8±4.5% for 48 and 72 hours, respectively) and 0-hour post-thaw (23.0±5.6% and 20.0±5.9% for 48 and 72 hours, respectively) mean PPM values.

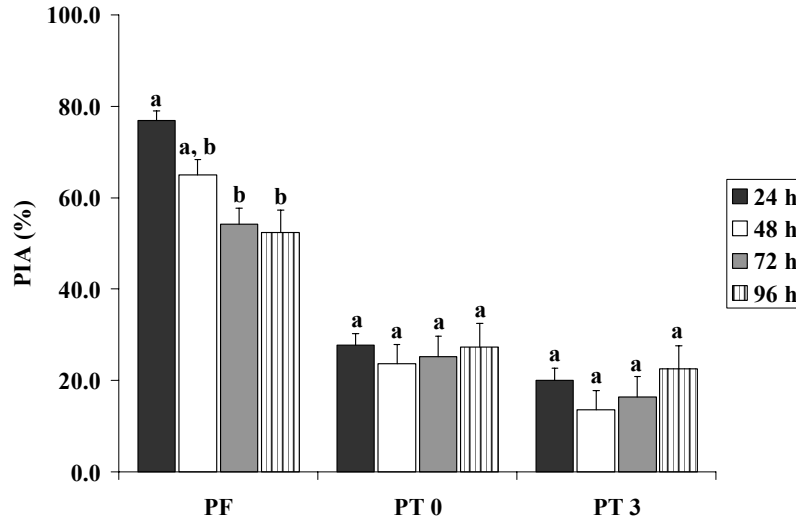


Figure 5.3. Mean (±SEM) percent acrosome intact sperm (PIA) at preefreeze (PF) and 0 and 3 hours post-thaw (PT) from cryopreserved bovine epididymal sperm stored in the cauda epididymidis of testes at 4°C for 24, 48, 72 or 96 hours (h). ^{a,b}Means with different superscripts within time of measurement are significantly different ($P \leq 0.0001$).

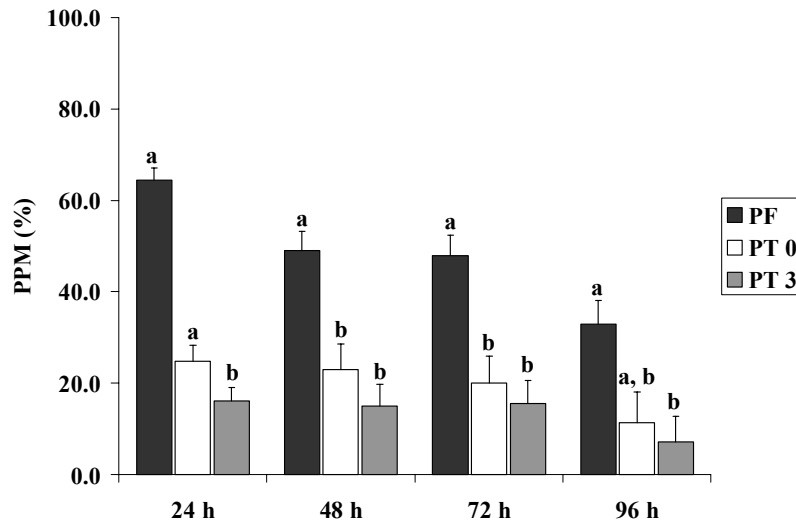


Figure 5.4. Mean (±SEM) percent progressive motility (PPM) from cryopreserved bovine epididymal sperm stored in the cauda epididymidis of testes at 4°C for 24, 48, 72 or 96 hours (h). ^{a,b}Means with different superscripts within the 48-hour ($P=0.0021$) and 72-hour ($P=0.0026$) storage-time treatment groups are significantly different between preefreeze (PF) and post-thaw (PT) 0 h. ^{a,b}Means with different superscripts within the 48-hour and 72-hour cool storage-time treatment groups are significantly different between preefreeze and 3 hours post-thaw ($P < 0.0001$). ^{a,b}Means with different superscripts within the 24-hour ($P < 0.0001$) and 96-hour ($P=0.0152$) storage-time treatment groups are significantly different between preefreeze and 3 hours post-thaw. ^{a,b}Means with different superscripts within the 24-hour cool storage-time treatment group are significantly different between 0 hour post-thaw and 3 hours post-thaw ($P=0.0003$).

The 48-hour and the 72-hour cool storage-time treatment groups also had a significant difference ($P<0.0001$) between mean prefreeze ($49.0\pm4.2\%$ and $47.8\pm4.5\%$ for 48 and 72 hours, respectively) and 3-hours post-thaw ($15.0\pm4.7\%$ and $15.6\pm5.0\%$ for 48 and 72 hours, respectively) mean PPM values. Differences ($P<0.0001$ and $P=0.0152$, respectively) for the cryopreserved samples from the 24- and 96-hour cool storage-time treatment groups were also noted between mean prefreeze ($64.4\pm2.6\%$ and $32.9\pm5.1\%$ for 24 and 96 hours, respectively) and 3-hours post-thaw ($16.2\pm2.9\%$ and $7.1\pm5.6\%$ for 24 and 96 hours, respectively) mean PPM values. Only cryopreserved samples from the 24-hour cool storage-time treatment group were found to be significantly different ($P=0.0003$) between the mean PPM values for 0 hour post-thaw ($24.8\pm3.5\%$) and 3 hours post-thaw ($16.2\pm2.9\%$) indicating that there had been a significant amount of latent cryoinjury to the epididymal sperm.

Significant differences were also detected from prefreeze to post-thaw for PIA in all cool storage-time treatment groups confirming the loss due to cryodamage (Figure 5.5). Differences were found between prefreeze ($64.9\pm3.4\%$, $54.1\pm3.6\%$ and $52.3\pm5.0\%$ for 48, 72 and 96 hours, respectively) and 0-hour post-thaw ($23.6\pm4.2\%$, $25.2\pm4.5\%$ and $27.2\pm5.1\%$ for 48, 72 and 96 hours, respectively) mean PIA values for the cryopreserved sperm that had been stored within the testicle for 48, 72 and 96 hours ($P<0.0001$, $P=0.0009$ and $P=0.0269$, respectively). All cool storage-time treatment groups 24 through 96 hours also were found to be significantly different ($P<0.0001$ for 24 through 72 hours and $P=0.0085$ for 96 hours) between prefreeze ($76.9\pm2.1\%$, $64.9\pm3.4\%$, $54.1\pm3.6\%$ and $52.3\pm5\%$ for 24, 48, 72 and 96 hours, respectively) and 3-hours post-thaw ($20.1\pm2.6\%$, $13.6\pm4.2\%$, $16.3\pm4.4\%$ and $22.6\pm5.0\%$ for 24, 48, 72 and 96 hours, respectively) mean PIA values.

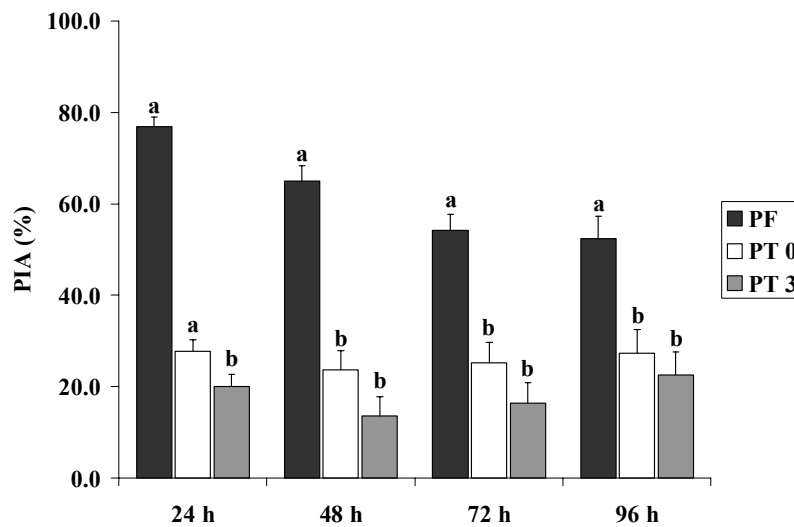


Figure 5.5. Mean (\pm SEM) percent sperm with intact acrosomes (PIA) from cryopreserved bovine epididymal sperm stored in the cauda epididymidis of testes at 4°C for 24, 48, 72 or 96 hours (h). ^{a,b}Means with different superscripts within the 48-hour ($P<0.0001$), 72-hour ($P=0.0009$) and 96-hour ($P=0.0260$) storage-time treatment groups are significantly different between preefreeze (PF) and 0 hour post-thaw (PT). ^{a,b}Means with different superscripts within the 24-, 48- and 72- hour ($P<0.0001$) and 96-hour ($P=0.0085$) storage-time treatment groups are significantly different between preefreeze and 3 hours post-thaw.

Analysis of the number of sperm with a retained cytoplasmic droplet (using an eosin B/fast green stain) from preefreeze and 0 hour post-thaw was completed. The mean percent of sperm with retained cytoplasmic droplets at preefreeze ($37.9 \pm 2.6\%$, $28.9 \pm 4.2\%$, $36.7 \pm 4.4\%$ and 30.6 ± 5.0 for 24, 48, 72 and 96 hours, respectively) and 0 hour post-thaw ($6.0 \pm 1.0\%$, $4.5 \pm 1.6\%$, $7.2 \pm 1.7\%$ and 8.2 ± 1.9 for 24, 48, 72 and 96 hours, respectively) were significantly different ($P < 0.0001$).

Materials and Methods (IVF)

Experimental Design (IVF)

Cryopreserved samples of bovine epididymal sperm were collected during the Cool Storage Experiment in Chapter 5. Epididymal sperm (total of 24 units) from three males with a preefreeze motility of greater than 70% from the 24-hour and the 48-hour cool storage-time treatment groups were used for the IVF Experiment in Chapter 5. Epididymal sperm from these three bulls and one batch of frozen ejaculated semen from one bull (Holstein) of known IVF fertility (ID no. 7H3429) (control sperm) were subjected to a standard IVF protocol modified by BOMED, Inc. (Ball et al., 1983; Parrish et al., 1985, 1986; Sirard et al., 1988). The ejaculated frozen-thawed semen has been used as the bovine IVF standard in this laboratory. Embryos resulting from IVF were cultured and development was evaluated at 24-hour intervals for up to 9 days of incubation.

Experimental Procedure (IVF)

Sperm TL medium (Parrish et al., 1988) was prepared with the addition of 0.006 g/ml of bovine serum albumin (Sigma, St. Louis, MO), 1mM sodium pyruvate and 25 μ g/ml of gentamicin (GibcoBRL, Grand Island, NY). Modified Tyrode-Lactate medium with HEPES (mTL-HEPES) was prepared by adding 0.003 g/ml of bovine serum albumin, 0.2mM sodium pyruvate and 25 μ g/ml of gentamicin (Bavister et al., 1983). Sperm TL

medium and mTL-HEPES were warmed in a water bath to 39°C prior to use with either epididymal sperm or bovine oocytes. IVF medium (modified Tyrode-Lactate medium) was modified with 0.006 g/ml of bovine serum albumin (fatty-acid free, Sigma, St. Louis, MO), 0.2mM sodium pyruvate and 25 µg/ml of gentamicin (Bavister and Yanagimachi, 1977; Bavister et al., 1983). Starting 3 hours prior to IVF procedure, the IVF medium was placed into an incubator maintained at 39°C in humidified air with 5% CO₂ for equilibration. For in vitro culture, CR1aa medium (Rosenkrans et al., 1993) was made and equilibrated in this laboratory on day of its use.

Bovine abattoir oocytes were collected and shipped overnight via FedEx at 39°C in maturation medium (BOMED, Madison, WI). Oocytes were removed from maturation medium and transferred to mTL-HEPES for further processing and examination. All of the oocytes (n=615) in the vials were combined to reduce oocyte group effects. Grade-1 oocytes, as defined by DeLoos et al. (1989), were washed four times through IVF medium before randomly placing groups of 10 to 12 oocytes in to 46-µl microdrops overlaid with warm mineral oil.

Sperm were thawed for 1 minute in a 39°C water bath. Thawed specimens were expelled from their 0.5-ml straws into 15-ml plastic conical tubes containing 3 ml of Sperm TL medium. Samples were then centrifuged at 700 x g for 6 minutes to remove sperm from the glycerol. After centrifugation, the supernatant was removed from the sperm pellet and subsequently resuspended in 2 ml of Sperm TL medium. Samples were washed again before making a final sperm suspension of 2 ml with Sperm TL medium. Small aliquots were removed from the samples to assess sperm post-thaw motility.

Prior to the IVF procedure, the sperm suspension (50 µl) was placed into 46-µl droplets containing oocytes. After the addition of sperm each droplet received 4 µl of

heparin stock solution (5 mg/ml) (Sigma, St.Louis, MO) to aid the sperm preparation for IVF. Sperm/oocyte droplets (total of 100 μ l) were returned to the incubator and maintained at 39°C in humidified air with 5% CO₂ for 18 hours. At the conclusion of 18 hours, the oocytes were removed from the sperm solution and washed to remove cellular debris. Each oocyte was washed two times through 10% modified TL-HEPES and four times through CR1aa medium before resting in groups of 20 in 35- μ l drops of CR1aa medium under warm mineral oil. Culture droplets with sperm-exposed ova were maintained in the incubator (39°C) with 5% CO₂ for the remainder of the culture period. Culture medium was refreshed every third day by washing the droplets two times with 25 μ l of fresh CR1aa medium. Embryo development was assessed on a 24-hour basis and morphological changes recorded for up to 9 days of incubation. There were 8 replicates of oocytes used in this experiment.

Statistical Analysis (IVF)

Data analysis was completed using contingency tables for each 24-hour period of embryo development to determine differences among cool storage-time treatment groups within each epididymal sperm sample compared with the control sperm treatment group. Each of the three males used for this experiment produced varied IVF results. A statistical method (meta analysis) was used that allowed these data to be adjusted for comparison (Hedges and Olkin, 1985). To adjust for the male affect the P values of each contingency table were calculated two times the natural logarithm and summed for the overall P value. This created a model with 6 degrees of freedom and allowed for comparison of each male as a proportion of the total.

Results (IVF)

Embryo development in vitro throughout the experiment is shown in Table 5.2. In vitro fertilization using frozen-thawed sperm from a bull (no. 7H3429) of known IVF fertility had a fertilization rate of 82.6% and blastocyst development rate of 34.9% throughout the experiment (control).

Bovine epididymal sperm stored at 4°C for 24 hours in the cauda epididymidis of testicles (n=6) had a fertilization rate (cleavage) of 26.1%, which was significantly lower ($P<0.05$) than that of the IVF control sperm (82.6%). Results from the 48-hour cool storage-time samples (n=6) had less promising results. Mean fertilization rate of 22.6% was significantly lower ($P<0.05$) than both IVF control sperm (82.6%) and 24-hour cool storage epididymal sperm (26.1%).

A slightly different pattern of embryonic developmental pattern occurred with embryos inseminated with control IVF sperm, 24-hour and 48-hour cool storage epididymal sperm at the 4-cell stage (67.9, 16.9 and 17.9%, respectively). A significant difference was noted between the control IVF sperm and both 24-hour and 48-hour cool

Table 5.2. Bovine embryo development after in vitro fertilization with frozen-thawed bovine epididymal sperm stored in the cauda epididymidis of testicles at 4°C for either 24-hour or 48-hour treatment periods prior to in vitro fertilization

Trt Group	n	Embryo Development (%)					
		2-cell	4-cell	8-cell	16-cell	Morula	BLST*
Control	109	90 (82.6) ^a	74 (67.9) ^a	62 (56.9) ^a	57 (52.3) ^a	47 (43.1) ^a	38 (34.9) ^a
24 h	249	65 (26.1) ^b	42 (16.9) ^b	27 (10.8) ^b	17 (6.8) ^b	16 (6.4) ^b	14 (5.6) ^b
48 h	257	58 (22.6) ^c	46 (17.9) ^b	30 (11.7) ^b	12 (4.7) ^c	6 (2.3) ^c	5 (1.9) ^c

^{a,b,c} Means with different superscripts are significantly different ($P<0.05$).

* BLST = Blastocyst.

storage-time treatment epididymal sperm ($P<0.05$). No difference was detected between the 24-hour and the 48-hour cool storage-time sperm treatment groups.

The embryo development to the 8-cell stage exhibited the same developmental pattern as the 4-cell stage. The embryo development rate to the 8-cell stage for control IVF sperm, the 24-hour and the 48-hour cool storage-time sperm treatment groups were 56.9, 10.8 and 11.7%, respectively.

On day 4 of in vitro culture embryo development rates (16-cell stage) for the control IVF sperm, the 24-hour and the 48-hour cool storage-time sperm treatment groups were 52.3, 6.8 and 4.7%, respectively. The IVF control sperm developmental rates were significantly greater than both the 24-hour and the 48-hour cool storage-time sperm treatment groups ($P<0.05$). A significant difference ($P<0.05$) was also detected between the 24-hour and the 48-hour cool storage-time sperm treatment group.

Evaluation of embryo development to the morula stage revealed rates of 43.1, 6.4 and 2.3% for control IVF sperm, the 24-hour and the 48-hour cool storage-time sperm treatment groups, respectively. The control IVF sperm treatment embryo development rate was significantly greater ($P<0.05$) than the development rate of the embryos from the 24-hour and the 48-hour cool storage-time sperm treatment groups. A significant difference ($P<0.05$) was also detected between the 24-hour and 48-hour cooled storage epididymal sperm embryo development rates at the morula stage of development.

Embryonic development rate to the blastocyst stage (assessed on day 6 of in vitro culture) was significantly greater ($P<0.05$) for the control IVF sperm treatment group (34.9%) compared with the 24-hour cool storage-time sperm treatment group (5.6%). There was development to the blastocyst stage (1.9%) for the embryos inseminated with epididymal sperm from the 48-hour cool storage-time treatment group and it was

significantly lower ($P<0.05$) than the blastocyst development rate of the 24-hour cool storage-time sperm treatment group.

Discussion

Results from preliminary studies on postmortem sperm collection that were reported for the mouse (Kishikawa et al., 1999; Stilley, 2002), the rat (Chapter 2), the dog (Stilley et al., 1999, 2000; Yu and Leibo, 2002), the goat (Chapter 3), the boar (Kikuchi et al., 1998) and the stallion (Bruemmer et al., 2002; James et al., 2002) were found to have similar epididymal sperm prefreeze parameter patterns as found in this study on the bull. The domestic bull had a gradual decline of epididymal sperm parameters evaluated after storage within the epididymis of testicles stored at 4°C for up to 96 hours postmortem.

The results from the Cool Storage Experiment in Chapter 5 suggest that the domestic bovine model would correlate with that of endangered species most closely related to the *Bos taurus* species. The PPM sperm values had an immediate decline from 24 to 48 hours and then again from 72 to 96 hours, as postmortem epididymal deterioration occurred in cold storage at 4°C. Percent progressively motile values started at 64.8% at 24 hours and declined to 30% at 96 hours of cold storage. These PPM values are greater than those reported by Bezuidenhout et al. (1995), Friedmann et al. (2000) and Kilian et al. (2000) for African buffalo epididymal sperm maintained within the epididymis under similar conditions and by Foote (2000) for domestic bovine tracts maintained at 5°C for 24-hour to 48-hour periods. Kikuchi et al. (1998) reported that motility of epididymal sperm collected postmortem from boar epididymides in cool storage (4°C) exhibited a similar decline of 60% at 24 hours to 37% at 72 hours as observed in this experiment.

The decline in PIA for bovine sperm from 24 to 96 hours in the present study was much more gradual than the PPM during the same 24-hour to 96-hour interval. As noted

for PPM from the 24-hour to the 48-hour cool storage-time treatments, there was also an immediate decline in PIA. From 48 to 96 hours the sperm maintained the same PIA pattern, with only a gradual decreasing trend in PIA values throughout the cool storage-time interval. This more gradual decrease in PIA suggests that the samples could be harvested and possibly used to create new offspring with the use of assisted reproductive technologies. The PIA values in this experiment were lower than those values reported by Kikuchi et al. (1998) for porcine epididymal sperm. The sperm stored in bovine epididymides for up to 3 days postmortem at 4°C were found to have a greater decrease in the number of sperm with intact acrosomes and plasma membranes than the boar.

In the Cryopreservation Experiment in Chapter 5, prefreeze PPM and PIA differences (readjusted analysis) among the initial cool storage-time treatment groups of 24, 48, 72 and 96 hours of storage in the cauda epididymidis of testicles at 4°C showed a similar downward trend as those in the Cool Storage Experiment in Chapter 5. Percent progressively motile bovine epididymal sperm was only significantly different between the 24-hour and 96-hour cool storage-time treatment groups at the prefreeze time measurement. There was an earlier decrease in the downward pattern found for PIA where the 24-hour cool storage-time treatment group was different from 72 and 96 hours at the prefreeze time measurement. The decrease in PPM and PIA values at the prefreeze time measurement from 24 hours through to 96 hours of the cool storage-time treatment could be explained as damage to the epididymal sperm caused either by cold shock during the 4°C storage or apoptosis as a part of the necrosis of the testicular tissue following excision of the testes from the male. In general, the decreasing PPM and PIA pattern from 24-hour through to the 96-hour cool storage-time treatment groups is similar in other species, such as the mouse (Stilley, 2002), the rat (Chapter 2), the dog (Stilley et al., 1999, 2000), the

goat (Chapter 3) the horse (Lubbe et al., 2000; Bruemmer et al., 2002; James et al., 2002) and Sika deer (Hishinuma et al., 2003).

In the present study, when making comparisons among the 24 hours through 96 hours of cool storage at 4°C in the cauda epididymidis, no significant differences were found among the thawed samples at either the 0-hour or the 3-hours post-thaw time measurement. This same pattern of sperm post-thaw survival among samples previously kept in cool storage within the epididymis of the testicles was also noted for both the goat (Chapter 3) and the stallion (Chapter 4). This suggests that cryopreservation is an equalizer when selecting bovine epididymal sperm that will survive cool storage over extended periods of time. A downward trend in the PPM and the PIA patterns was apparent from 24 hours to 96 hours of cool storage, however, the number of surviving sperm were equal among the cool storage-time treatment groups post-thaw.

Cryopreserved bovine epididymal sperm that had been previously cool stored in the epididymis for 24 hours postmortem at 4°C did not show initial damage at 0 hour post-thaw. The loss of motility noted in our study was slightly greater than that observed with cryopreserved epididymal sperm of the African buffalo (Kilian et al., 2000). Kikuchi et al. (1998) also reported greater loss of motility than the domestic bull with frozen-thawed porcine epididymal sperm that had been harvested from epididymides stored at 4°C for 24 hours postmortem. After 3 hours of co-incubation with Sperm TL medium the cryoinjuries became apparent in the bull. This later decline in both the PPM and the PIA patterns from 0 hour to 3 hours post-thaw has been previously described by Chandler et al. (1978), Fleming et al. (1976) and Saacke (1970) as latent cryoinjury, and is indicative of lower fertility for sperm from bulls that have these traits.

Cryopreserved bovine epididymal sperm from the 48-hour and the 72-hour cool storage-time treatment groups were found to have cryoinjury immediately at 0 hour post-thaw. The mean PPM and PIA values for both groups exhibited the same decline from prefreeze to 0 hour post-thaw. This is unlike the results reported for both the stallion (Chapter 4) and the gaur bull (Hopkins et al., 1988) at 72 hours and 96 hours of cool storage-time treatment. In both of these studies there was an initial increase in post-thaw PPM from the prefreeze values. No difference was noted between 0 hour and 3 hours post-thaw for mean PPM and PIA in either group indicating that the damage had been immediate and no latent injuries materialized at 3 hours post-thaw. Again, this result is unlike that reported for the stallion (Chapter 4) or the gaur bull (Hopkins et al., 1988). The PPM values in the present study that had initially increased at 0 hour post-thaw from prefreeze showed a decreased PPM at 3 hours post-thaw from the 0 hour post-thaw values. Kikuchi et al. (1998) have reported that the loss of motility from prefreeze to post-thaw was less dramatic for boar epididymal sperm preserved after postmortem storage at 4°C for 48 and 72 hours. Kikuchi et al. (1998) also proposed that this could be related to the lower prefreeze values reported for these two storage-time treatment groups for the domestic boar.

The 96-hour cool storage-time treatment group had a mean PPM value that was an additive effect of the 24-hour, the 48-hour and the 72-hour cool storage-time treatment groups. There was no difference initially upon thaw at 0 hour from the prefreeze PPM value. There was also no difference between mean PPM values at 0 hour post-thaw and 3 hours post-thaw. A difference occurred between the prefreeze mean PPM value and the 3-hours post-thaw value. The pattern of motility for the 96-hour cool storage-time treatment group for cryopreserved bovine epididymal sperm was different from the pattern noted for

equine epididymal sperm observed at the same cool storage-time treatments (the Cool Storage Experiment in Chapter 4). The cryopreserved equine epididymal sperm did not exhibit any differences from prefreeze through to 3 hours post-thaw.

Saacke (1970), Fleming et al. (1976) and Chandler et al. (1978) reported that PPM of cryopreserved bull sperm after 3 hours of incubation at 37°C post-thaw could be correlated with fertility when used for AI. If the correlation remains the same for cryopreserved bovine epididymal sperm, then the sperm retrieved after 96 hours of cool storage would have to be used with more aggressive methods of assisted reproductive technologies than AI. Goto et al. (1990) and Goto (1993) have used cryopreserved epididymal sperm to produce two live calves with ICSI.

Mean values for PIA in the 96-hour cool storage-time treatment group decline immediately from prefreeze to 0 hour post-thaw as noted for the 48-hour and 72-hour cool storage-time treatment groups. Hishinuma et al. (2003) reported that both PPM and viability of frozen-thawed epididymal sperm collected from Sika deer decreased when they were collected from cold stored epididymides. Also, as for the 48-hour and 72-hour cool storage-time treatment group, the mean PIA values between 0 hour and 3 hours post-thaw were not significantly different. This creates a similar pattern noted in the other three cool storage-time treatment groups with differences occurring at different time points depending on the amount of storage time within the testicle before the cauda epididymal sperm were frozen. Like PPM, Saacke (1970), Fleming et al. (1976) and Chandler et al. (1978) have also reported that PIA is correlated with fertility of cryopreserved bovine sperm used for AI. Because the epididymal sperm from the 96-hour cool storage-time treatment group do not show a decline from 0 hour post-thaw to 3 hours post-thaw their efficacy for use in fertilization is worthy of further study.

The bovine caudal epididymal sperm that were harvested for this experiment included an overall mean of 33.5% of sperm with attached cytoplasmic droplets at prefreeze analysis. Amann et al. (2000) reported that bulls with a high percentage of cytoplasmic droplets (>30%) attached to ejaculated sperm resulted in compromised fertility for IVF. In our study, at the 0-hour post-thaw analysis the mean number of sperm still retaining a cytoplasmic droplet had declined to 6.5%. Hopkins et al. (1988) indicated that the loss of attached cytoplasmic droplets after the freeze-thaw process was due to osmotic differences created by the addition of glycerol, freezing and/or thawing. Assessment of the decrease in the percentage of retained cytoplasmic droplets from prefreeze to post-thaw may be a means of evaluating epididymal sperm damage caused by the freezing and thawing process. This significant drop, across all cool storage-time treatment groups in our study suggests equal cryoinjury was sustained by all sperm samples.

Overall, these downward trends in sperm parameters in the Cryopreservation Experiment in Chapter 5 for all four cool storage-time treatment groups using bovine epididymal sperm indicates that cryopreservation of cooled epididymal sperm accounts for a large amount of damage to the sperm (~50% loss). Even with the damage induced by cryopreservation, there are still enough sperm that are either motile or retain membrane integrity and are likely capable of being used in conjunction with assisted reproductive technologies, such as IVF or ICSI to produce viable blastocysts and possibly offspring (Goto, 1993; Anel et al., 2000).

Results from IVF in the IVF Experiment in Chapter 5 indicated that with epididymal sperm from 24- or 48-hour cool storage-time treatment groups there were some initial difficulties achieving in vitro fertilization. While the control group sperm achieved

fertilization rates of 82.6%, a rate comparable to expected fertilization rates for bovine IVF (Zhang et al., 1992), both 24-hour and 48-hour cool storage-time treatment groups had initial fertilization rates of 26.1 and 22.6%, respectively. Pushett et al. (2000) found similar fertilization rates with domestic feline frozen-thawed epididymal sperm and IVF (30.8%). In our study, both 24-hour and 48-hour cool storage-time treatment groups were only 25% of the fertilization rate for the control IVF sperm (82.6%). This suggests that the epididymal sperm were damaged during the cryopreservation process or that the sperm were missing some factor(s) that could have been gained through the ejaculation process. Kikuchi et al. (1998) reported IVF rates for frozen-thawed porcine epididymal sperm from the 24-hour and 48-hour cooled storage treatments to be 30% of the control fresh epididymal sperm (40%).

In our study, post-thaw PPM values of 76, 55 and 45% for control sperm and 24-hour and 48-hour cooled storage sperm, respectively, indicate that there was a marked loss of motility after cryopreservation and thawing. The post-thaw PPM values indicated that the low fertilization rates are most likely due to cryodamage to the epididymal sperm. To increase fertilization rates of cryopreserved epididymal sperm, it is proposed that more research needs to be completed to determine the best cooling procedure to maintain sperm fertilizing ability. Another possibility would be the addition of other chemical components, such as adding progesterone to the maturation medium to enhance sperm performance, as has been reported in mice (Kholkute et al., 1995).

Another failure during the embryo development following the use of bovine epididymal sperm was at the time of genomic activation (8-cell to 16-cell stage). The percent of embryos developing to the 8-cell stage for 24-hour and 48-hour cool storage-time treatment groups were only 10.8 and 11.7%, respectively. These values were a 20%

of the 8-cell embryo development rate (56.9%) in the control IVF treatment group. Furthermore, the developmental loss from fertilization to the 8-cell stage was 31.1, 58.6 and 48.2% for the control, the 24-hour and the 48-hour cool storage-time treatment groups, respectively. The higher loss during embryo development is likely another indicator of damage or defect of the epididymal sperm. Cryodamaged sperm could have achieved fertilization but damage to the sperm DNA could prevent proper genomic activation at the 8-cell stage of embryo development, thus causing the embryos to cease development.

Cryopreserved or cooled ovine sperm has also been reported to have a decreased rate of embryonic development compared with fresh sperm (Maxwell and Salamon, 1993). This decreased embryonic development could be the result of DNA damage caused by either sperm ageing or cryodamage. Bladou et al. (1991) also reported a high incidence of embryo degeneration (>50%) for embryos produced by IVF with epididymal sperm from infertile human males.

The last area of embryonic development in which the epididymal sperm resulted in poor results was blastocyst development in vitro. The low bovine blastocyst development rate in this study (5.6% for 24-hour and 1.9% for 48-hour cool storage-time treatment groups) was comparable with 2% reported for porcine embryos produced by IVF with frozen-thawed epididymal sperm collected postmortem after 24 hours of cool storage in the epididymides (Kikuchi et al., 1998). All bovine embryos that developed to the blastocyst stage in our study did hatch by day 8 of in vitro culture. All of the hatched blastocysts had potential to produce offspring had they been transferred to recipient cattle. Khorram et al. (2000) found that human embryos that hatched by day 6 (correlates to day 8 in cattle) of in vitro culture had a higher implantation potential than IVF-derived blastocysts that failed to hatch.

The poor development to the blastocyst stage of embryos created using the cryopreserved bovine epididymal sperm from the 24-hour and the 48-hour cool storage-time treatment groups compared with the control sperm group (34.9%), however, brings into question the normality of the embryos in culture as well as the efficacy of the embryo culture system. Embryonic loss in the IVF control sperm was not high enough to cause suspicion of the embryo culture system. Also, the fertilization and embryo developmental rates of the control IVF sperm were comparable with and, in some cases, better than other bovine IVF studies (Lonergan et al., 1994; Thundathil et al., 2001). This leaves the question of embryonic normality that again leads to possible damage of sperm prior to fertilization. The blastocyst development rate of cooled stored epididymal sperm derived embryos indicates that much research is still needed to develop efficient procedures for using cryopreserved epididymal sperm to produce IVF-derived embryos.

Findings from the IVF Experiment in Chapter 5 have verified that IVF is possible with epididymal sperm collected postmortem from mature bulls. The number of blastocysts produced was low but each blastocyst produced could have potentially produced a live offspring. More research is needed to improve the methodology so that more consistent and better results can be obtained from epididymal sperm collected and subjected to the IVF procedure. For those samples collected after 24 hours postmortem, a more efficient use of these gametes might be to use ICSI for the creation of blastocysts and subsequent offspring. Calves have been born after using ICSI to produce embryos with both epididymal sperm (Goto et al., 1990; Goto, 1993) as well as with ejaculated sperm (Hamano et al., 1999).

Intracytoplasmic sperm injection was not attempted for this series of experiments because of the current difficulties achieving fertilization or the production of offspring with

ICSI in the bovine species. Goto (1993) reported a blastocyst development rate of 7.3% for bovine embryos created by ICSI with frozen-thawed epididymal sperm. Only a few offspring have been produced to date by this approach in cattle (Goto et al., 1990; Goto, 1993; Hamano et al., 1999).

Various research groups have reported the possible use of ICSI and demonstrated success with the use of epididymal sperm from mice in the production of pups (Kishikawa et al., 1999) and for cats in the production of blastocysts (Pushett et al., 2000). The former study involved epididymal sperm collected 20 days postmortem, while the latter study used frozen/thawed epididymal sperm collected ranging from 5 to 24 hours postcastration.

Shaw et al. (1995) have reported the production of embryos using fresh epididymal sperm collected <24 hours postmortem from African buffalo bulls using IVF and CR1aa medium in the culture system. The study showed a higher fertilization rate and development rate to the morula stage of embryo development, however, none of these embryos continued on to blastulation. Of all the bovine embryos that developed to the blastocyst stage in the IVF Experiment in Chapter 5, 100% hatched by day-8 post-insemination in both the 24-hour and 48-hour cool storage-time treatment group derived embryos.

Shaw et al. (1995) reported higher fertilization and development rates in buffalo likely because the epididymal sperm used was not frozen and therefore, was not exposed to additional membrane damage as that of the current study using cryopreserved domestic bull epididymal sperm. Epididymal sperm used in the IVF Experiment in Chapter 5 were also obtained from testicles in cool storage for 24-hours and 48-hours postmortem and therefore, exposed to longer time periods of potential damage from necrosing tissue. Perhaps better IVF results could have been obtained if the sperm had not been

cryopreserved and had been collected for use prior to 24 hours postmortem. This finding should be taken into consideration for future research with domestic bull sperm.

Conclusion

In these experiments domestic bulls were used to determine the status of sperm stored in the epididymides of testicles stored at 4°C for 24 to 96 hours. The results of these studies show that sperm can withstand storage postmortem prior to harvest and for the potential use with assisted reproductive technologies. These results can help to create an animal model beneficial for use in closely related exotic species. Instead of wasting precious germplasm to determine the correct “window of opportunity” or methods to be used for sperm banking, the domestic bull should be used in this capacity. These results indicate that sperm can be transported within the epididymides of the testicles stored at 4°C for up to 96 hours postmortem with only a limited loss of motility and/or acrosomal integrity.

These experiments have also found that epididymal sperm procured from the cauda epididymidis stored at 4°C for 24, 48, 72 or 96 hours can undergo the freeze-thaw process and still produce motile and viable sperm. In the short-term cooled storage treatments in our study the number of motile and/or viable sperm may be relatively low but with the use of IVF and ICSI even with a low number of sperm available could be used to produce embryos, and possibly live offspring.

Finally, our findings indicate that though the cool storage-time treatment groups are significantly different than control IVF values, frozen-thawed epididymal sperm can produce blastocysts and therefore, should be considered as a potential means of preserving genetic material. Future research should include fresh epididymal sperm and/or other methods of IVF that require less stressed epididymal sperm for embryo production. The

use of ICSI techniques should also been investigated to determine if the sperm collected >48 hours postmortem and cryopreserved can be used to create embryos and offspring. Once these issues have been evaluated in the domestic bull then more efficient research can be completed on various related exotic species, with the hope for the production of genetically valuable offspring.

CHAPTER 6

COMPARISON OF TWO COMMON CRYOPROTECTANT MEDIA FOR EPIDIDYMAL SPERM STORED IN THE EPIDIDYMIDES OF TESTES AT 4°C FOR 24 AND 48 HOURS FROM THE BOVINE AND FOR 24, 48, 72 AND 96 HOURS FROM THE CAPRINE AND EQUINE MALES

Introduction

Cryopreservation of epididymal sperm has become an area of great importance for assisted reproductive technologies. Cryopreserved epididymal sperm has been increasingly used over the last few years in the field of human infertility in conjunction with intracytoplasmic sperm injection (ICSI) and to a lesser extent with in vitro fertilization (IVF). Cryopreserving epididymal sperm has also become extremely important for reproductive research involving exotic or endangered species. With endangered species, males become even more genetically important and their gametes should be harvested to ensure the continuation of that species. Collection of epididymal sperm postmortem can help to ensure that valuable DNA can be used with assisted reproductive technologies to produce more genetically diverse offspring.

Previous experiments have found that among domestic bovine, caprine and equine males viable epididymal sperm can be collected up to 96 hours postmortem when the testes are maintained at 4°C (James et al., 2002; Chapters 3, 4 and 5). To increase the value of epididymal sperm collected postmortem, preservation methods need to be developed that can further increase the duration of sperm viability. Epididymal sperm recovered <6 hours postmortem or post-castration from cats (Pushett et al., 2000) and dogs (Stilley et al., 1999, 2000; Yu and Leibo, 2002), as well as, several African wild ruminants (Black wildebeest, blesbok, Roan antelope, gemsbok, nyala, eland and African buffalo) (Kilian et al., 2000), domestic stallions, zebra and rhinoceros (Lubbe et al., 2000) can be preserved by

cryopreservation with minimal loss of motility. Stilley et al. (2000) reported that epididymal sperm collected from dogs beyond 6 hours postmortem exhibited motility and viability after cryopreservation.

Live offspring have been produced by using cryopreserved epididymal sperm in conjunction with various forms of assisted reproductive technologies. Blash et al. (2000) reported the production of a live kid after using cryopreserved caprine epididymal sperm for artificial insemination (AI). Artificial insemination was also used to produce a live foal from cryopreserved equine epididymal sperm (Barker and Gandier, 1957). Bartels et al. (2001) reported on the live birth of an eland from cryopreserved epididymal sperm used in conjunction with AI.

The efficacy of various cryoprotective extenders in preserving these valuable gametes in a number of species has been studied. A study concentrating on Black wildebeest, blesbok, Roan antelope, gemsbok, nyala, eland and African buffalo examined the cryosurvivability afforded by two different cryoextenders (Kilian et al., 2000). It was determined that the extender that maintained the best post-thaw motility was Biladyl Fraction A + B when compared with Ham's F-10 + Triladyl. Lubbe et al. (2000) determined that there was no difference in post-thaw survival of epididymal sperm cryopreserved in Biladyl Fraction A (Minitube, Germany) when compared with a skim-milk based glycerol equine extender for freezing horse, zebra and rhinoceros sperm.

Epididymal sperm collected from impala was subjected to five different cryodiluents and stored at 28° to 32°C or 4 to 7°C for 8 to 16 hours and samples refrigerated for 8 hours were subsequently frozen (Rush et al., 1997). Those sperm stored ≤16 hours with glycerol exhibited the highest motility and a 64% sperm penetration rate in homologous mature oocytes.

Watson et al. (1997) reported on the efficacy of glycerol, ethanediol and dimethyl sulfoxide (DMSO) as cryoprotectants for epididymal sperm collected from the waterbuck, the Greater kudu and the warthog. In this study there were no overall toxic effects from glycerol or ethanediol, but DMSO was toxic to the Greater kudu sperm at the lowest temperature (4° to 7°C) and the waterbuck and the warthog sperm at the higher temperature (28° to 32°C) after refrigeration or incubation for 8 to 16 hours. When used for cryopreservation, glycerol was most effective while ethanediol was completely ineffective.

Schmid et al. (1997) also reported on cryodiluents for Greater kudu epididymal sperm. This study reported individual bull preference for different cryodiluents but overall the most effective medium was Biladyl with 7% glycerol. Research was completed comparing the longevity of epididymal sperm collected from a White rhinoceros in Biladyl with three different glycerol concentrations. The samples were kept at 4° to 10°C and assessed at 4, 8 and 12 hours after the addition of varied glycerol concentrations for motility and sperm acrosomal integrity. Williams et al. (1995) it was reported that the sperm maintained in the control medium (sperm TALP) was ideal for this form of storage because glycerol induced the acrosome reaction.

These experiments with epididymal sperm found that, as had been reported for ejaculated sperm, cryosensitivity is species dependent and that further study is needed to determine the best extenders to be used when cryopreserving the gametes of males for any species. This study was undertaken to make a comparison of two different cryoprotective media commonly used by each of the domestic bovine, caprine and equine males, to determine which, if any, would be the most beneficial for future preservation of epididymal sperm collected at postmortem.

Materials and Methods

Experimental Design

Testicular pairs were collected from mature bovine, caprine and equine males after slaughter and delivered overnight to the laboratory for these experiments. One of the testicles, of the pair, was randomly allotted to the cool storage-time treatment of 24 hours (control) while the other testicle was allotted to the 48, 72 or 96 hours of postmortem cool storage-time treatment groups and placed in storage at 4°C. Bovine testicular samples were only allotted to the 24-hour or the 48-hour cool storage-time treatment groups. After collection of epididymal sperm from bovine, caprine and equine testes each cooled epididymal sample was split to subsequently compare the efficacy of two cryoprotective media for the preservation of sperm.

The efficacy of the two different cryoprotective media were also compared across time of cool storage in the testes at 4°C for 24 and 48 hours in the bovine (n=30 testicles per cool storage-time treatment group and 2 epididymal sperm straws per testicle per cryoprotective media group) and 24, 48, 72 and 96 hours in the caprine (n=23, 8, 8 and 7 testicles per cool storage-time treatment groups, respectively and 2 epididymal sperm straws per testicle per cryoprotective media group) and equine (n=17, 6, 5 and 5 testicles per cool storage-time treatment group, respectively and 2 epididymal sperm straws per testicle per cryoprotective media group) males. The cryopreserved samples utilized for analysis in this experiment of bovine, caprine and equine epididymal sperm were collected during previous experiments (Chapters 3, 4 and 5, Section Cool Storage). Only testicular pairs that had 2 or more straws of each medium and stored in liquid nitrogen (LN₂) were used for this experiment. Epididymal sperm samples were thawed and assessed at 0 and 3 hours post-thaw. Each sperm analysis included the percent progressively motile (PPM)

and percent intact acrosome (PIA). Analysis also included percent of attached cytoplasmic droplets (CD) at prefreeze as well as 0 hour post-thaw.

Experimental Procedure

Mature bovine, caprine and equine testicle pairs were collected from an abattoir located in San Angelo, TX. Testicular pairs were individually packaged in Ziploc[®] bags, placed on ice to bring the temperature to 4°C and shipped in a Styrofoam[™] container overnight via FedEx to the Louisiana State University Embryo Biotechnology Laboratory.

Testicles were processed, in the following manner, for each cool storage-time treatment group. The testicle and extraneous tissue were brought to room temperature (25°C) from cool storage at 4°C in ≤ 30 minutes. During this warming period each testicle was dissected away from its tunica vaginalis as well as other extraneous tissue.

Medium (Sperm TL; Parrish et al., 1988) was prepared by adding 0.006 g/ml of bovine serum albumin (Sigma, St. Louis, MO), 1mM sodium pyruvate and 25 μ g/ml of gentamicin (GibcoBRL, Grand Island, NY) and warmed in a water bath to 39°C prior to use with epididymal sperm. The two cryoprotective media utilized for preservation of bovine and caprine samples, egg-yolk citrate with 7% glycerol (7 EYG) (Salisbury et al., 1941) and milk with 7% glycerol (7 MG) (Zaugg and Almquist, 1972) were prepared as one batch and frozen for subsequent use in 10-ml aliquots. Skim milk egg yolk with 4% glycerol (4 SM-EYG) (Varner et al., 1991) and egg-yolk citrate with 4% glycerol (4 EYG) extender for equine samples were also made in one batch and frozen as 10-ml aliquots. On day of use EYG and MG or SM-EYG was thawed at 4°C until liquefied and then warmed to room temperature (25°C).

Once dissected from the testis, the bovine, caprine and equine epididymides were minced and placed into a 60-mm tissue culture dish (Becton and Dickinson & Company,

Franklin Lakes, NJ). The tissue was rinsed with 3 ml of Sperm TL medium and stood for 5 minutes to allow sperm to escape from the epididymis. After 5 minutes, the tissue was removed and the remaining medium was pipetted into a 15-ml centrifuge plastic tube (Corning, Inc., Corning, NY). Minced equine epididymides were not allowed to stand for 5 minutes because of the overwhelming numbers of sperm released. In contrast, the minced equine tissue was rinsed and immediately processed.

The sperm were then centrifuged (Sorvall® Tabletop Refrigerated Centrifuge RT-6000, Sorvall® Centrifuges, Wilmington, DE) at 700 x g for 6 minutes to create a sperm pellet. Supernatant was removed and a 300×10^6 sperm/ml suspension was created by adding Sperm TL medium to the pellet. The sperm analysis was completed on a 10- μ l sample drawn from the suspension.

Sperm analysis included PPM, PIA, percentage of sperm retaining a cytoplasmic droplet and determination of sperm concentration. For assessment of PPM, PIA and the percentage of retained cytoplasmic droplets, a 5- μ l volume of the sperm suspension was added to 95 μ l of Sperm TL medium. Then, a 1:20 dilution was created by adding another 5 μ l sperm suspension sample to 95 μ l of distilled water to be used for a hemacytometer count. Motility was assessed on 10 μ l sampling of the 1:20 sperm dilution using a Nikon Labophot 100X phase contrast microscope (Nikon Instruments, Inc., Lewisville, TX).

Acrosomal integrity and percentage of sperm retaining a cytoplasmic droplet were assessed by examining an epididymal sperm specimen stained with eosin B/fast green (Aalseth and Saacke, 1986). A volume of 20 μ l from the 1:20 sperm solution was mixed with 10 μ l of eosin B/fast green stain. After co-incubating for 1 minute, the mixture was placed on a slide (10 μ l) and smeared and then the sperm were heat fixed. Each sample

was analyzed by counting 200 sperm and respective category was determined. Cells were categorized as viable if they retained sperm cell membrane integrity and excluded the dye. Those sperm that appeared pink or red were considered membrane compromised and thus, nonviable.

When motility and sperm number had been determined the rest of the sample was prepared for cryopreservation. Prior to final centrifugation the samples were split into two equal groups, with one portion being randomly assigned to one cryoprotective medium and the second portion to the other cryoprotective medium. Each sperm sample was centrifuged at 700 x g to obtain separation of sperm from Sperm TL medium. Sperm TL medium was removed from the pellet and part A (14% or 8% glycerol for bovine and caprine or equine sperm, respectively) of 7 EYG or 7 MG (bovine and caprine sperm) or 4 SM-EYG or 4 MG (equine sperm) extender was added to create a 30×10^6 sperm/ml dilution. This sperm dilution (bovine and caprine sperm) was cooled at 4°C for a total of 1 hour, while additions of part B (0% glycerol) 7 EYG or 7 MG were made at 15-minute increments (Figure 6.1). Each 15-minute period brought the addition of 0.125% of the total final volume. After 1 hour, the sperm solution was drawn into 0.5-ml plastic straws and moved to the freezing chamber. The time at 4°C for the equine samples was 2 hours and the increments of part B (0% glycerol) 4 SM-EYG or 4 MG addition were 30 minutes (Figure 6.1).

The straws were placed 2 cm above the level of LN₂ in the freezing chamber. The start temperature at 2 cm was -110°C. The freezing protocol for the bovine and caprine sperm required that the straws remain at this level for 8 minutes until they reached -100°C at which time they were rapidly frozen by immersion into the LN₂ at -196°C (Figure 6.2). The rate of freezing was -13°C/minute for the bovine and caprine epididymal sperm from

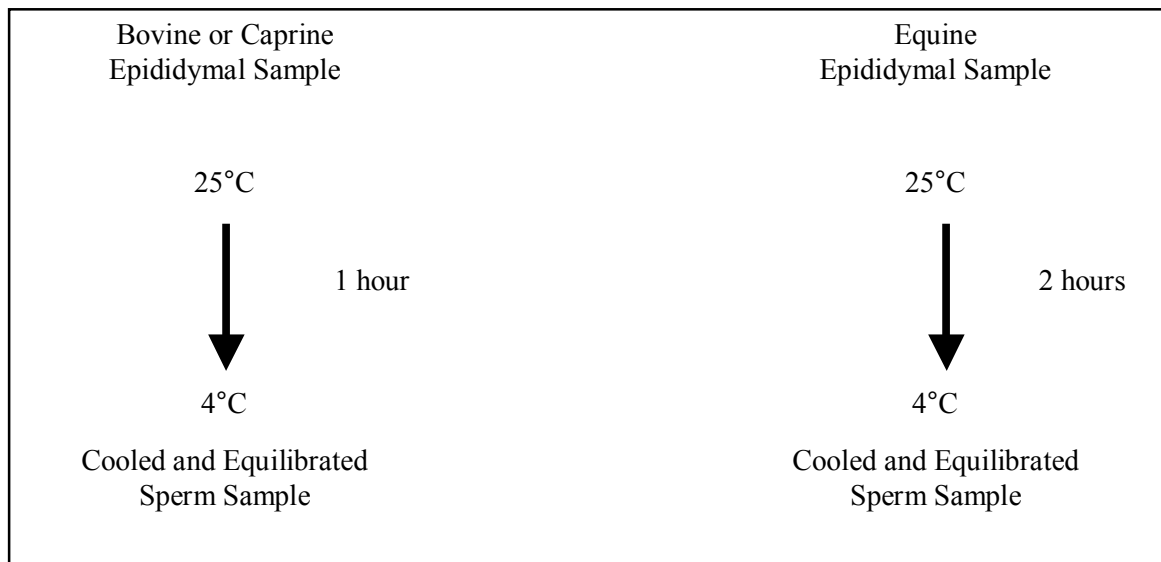


Figure 6.1. The cryopreservation equilibration protocol diagram for epididymal sperm harvested from cauda epididymidis of testes stored at 4°C for 24, 48, 72 and 96 hours postmortem from the bovine, caprine and equine males.

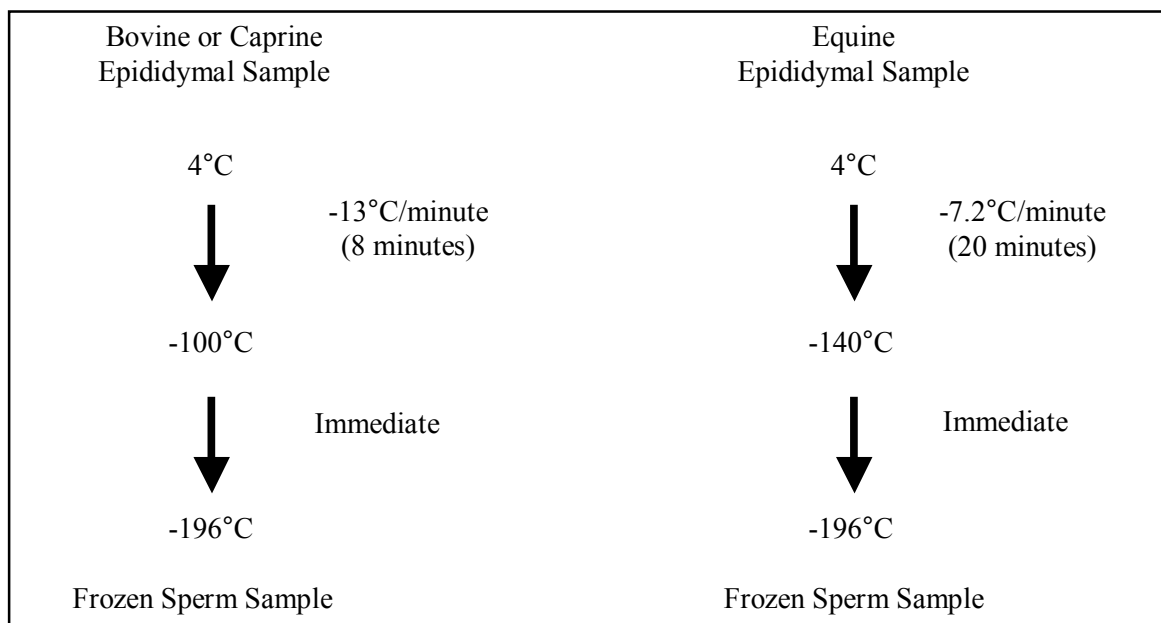


Figure 6.2. The freezing protocol diagram for epididymal sperm harvested from cauda epididymidis of testes stored at 4°C for 24, 48, 72 and 96 hours postmortem from the bovine, caprine and equine males.

4°C to -100°C. The freezing protocol for the equine epididymal sperm varied the time spent at 2 cm above the LN₂. Equine straws were kept at this level for 20 minutes until they reached -140°C and then immersed into the LN₂. The rate of freezing was -7.2°C/minute for the equine epididymal sperm from 4°C to -140°C (Figure 6.2).

Epididymal sperm were cryopreserved for at least 24 hours before thawing for analysis. On the day of analysis for the thawed sperm, Sperm TL medium was prepared and warmed to 39°C. Two straws from each epididymis were removed from LN₂ and placed into a 39°C water bath for 1 minute. The samples were combined in a 15-ml centrifuge tube with 3 ml of pre-warmed Sperm TL medium. The sperm mixture was centrifuged at 700 x g for 6 minutes and the supernatant removed from the sperm pellet. This pellet was then resuspended with another 3 ml of Sperm TL medium and washed once more. This process diluted the glycerol in the epididymal sperm. Sperm pellets were resuspended in 2 ml of Sperm TL medium and prepared for analysis. Analysis consisted of the PPM, PIA and the percent of retained cytoplasmic droplets taken at 0 hour post-thaw as well as PPM and PIA taken at 3 hours post-thaw. The analysis of PPM at 3 hours, as well as, PIA has been correlated with the fertility of cryopreserved bull sperm by Saacke (1970), Fleming et al. (1976) and Chandler et al. (1978).

Statistical Analysis

Bovine, caprine and equine sperm parameters (e.g., PPM, PIA and percent of retained cytoplasmic droplets) are reported as a mean value \pm SEM. Data were analyzed using Proc Mixed in SAS[®] (Version 8.0) (SAS, 1996) and a repeated measures design, with a Tukey multiple comparison adjustment. This analysis determined if there was a difference between media treatment groups and among cool storage-time treatment groups by examining the difference between the prefreeze and post-thaw PPM, PIA and

percentage of sperm with retained cytoplasmic droplets values. Also, the sperm post-thaw mean values were compared between the two different cryoprotective media used for these experiments.

Results

Bovine Epididymal Sperm Parameters from Samples Cryopreserved with Egg-Yolk Citrate Plus 7% Glycerol

Results indicated that PPM prefreeze for bovine epididymal sperm were not different between 24 and 48 hours (76.3 ± 3.5 and $64.7 \pm 3.5\%$, respectively) (Figure 6.3). Bovine epididymal sperm cryopreserved in 7% EYG shows mean PPM values for 24 and 48 hours at 0 hour post-thaw of 22.9 ± 3.7 and $24.7 \pm 3.7\%$, respectively. The post-thaw analysis made after 3 hours of incubation produced mean PPM values for 24- and 48-hour cool storage-time treatment groups of 14.5 ± 3.3 and $17.6 \pm 3.3\%$, respectively. No difference was detected between the mean values for PPM for either of the cool storage-time treatment groups at either 0 hour or 3 hours post-thaw.

Sperm PIA from prefreeze samples for 24 and 48 hours were 93.3 ± 3.6 and $86.3 \pm 3.6\%$, respectively (Figure 6.4). The mean PIA values at 0 hour post-thaw for the 24- and 48-hour cool storage-time treatment groups were 19.3 ± 3.1 and $22.0 \pm 3.1\%$, respectively. Bovine epididymal sperm PIA values at 3 hours post-thaw were 11.1 ± 2.3 and $14.3 \pm 2.3\%$ for 24 and 48 hours, respectively. No difference was detected between the mean values for PIA for either of the cool storage-time treatment groups at prefreeze, 0 hour or 3 hours post-thaw.

Cryopreserved (7% EYG) samples that had been stored in the cauda epididymidis for 24 hours were significantly different between prefreeze and 3-hours post-thaw values ($P < 0.0001$), as well as, between 0- and 3-hours post-thaw mean PPM values ($P = 0.002$)

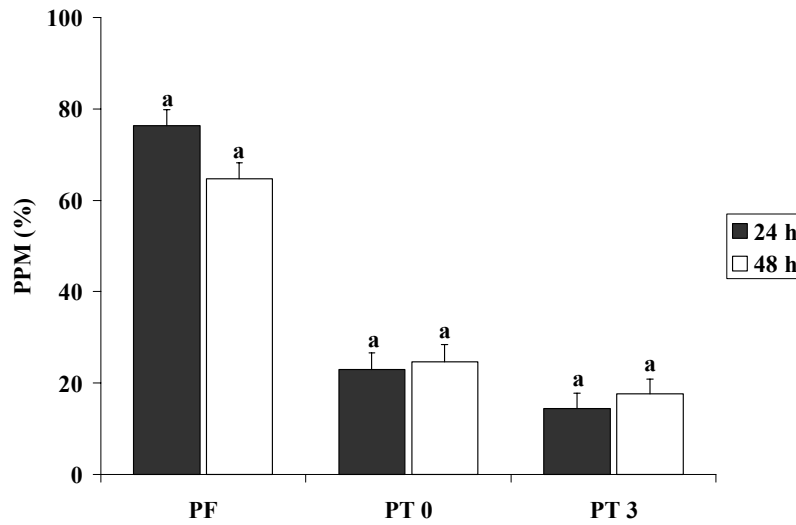


Figure 6.3. Mean (\pm SEM) percent progressive motility (PPM) at prefreeze (PF) and 0 and 3 hours post-thaw (PT) from samples cryopreserved with egg-yolk citrate plus 7% glycerol for bovine epididymal sperm previously stored in the cauda epididymidis of testes at 4°C for 24 hours or 48 hours (h). No significant differences ($P>0.05$).

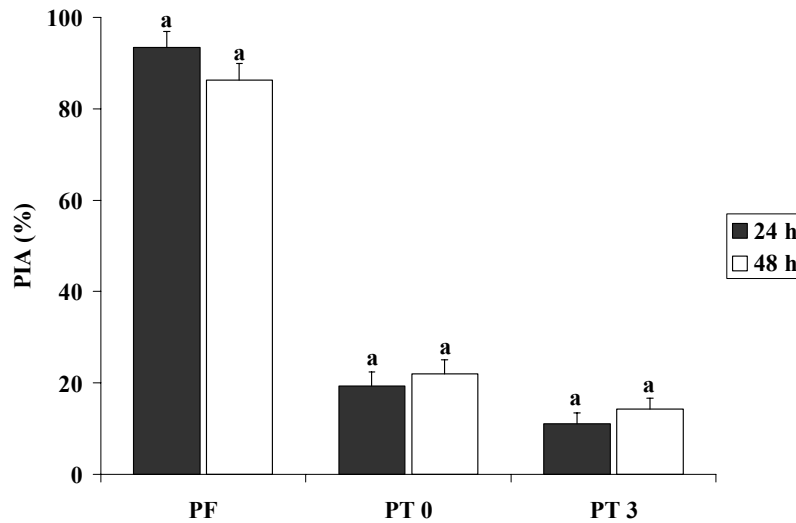


Figure 6.4. Mean (\pm SEM) percent sperm with acrosomal integrity (PIA) at prefreeze (PF) and 0 and 3 hour post-thaw (PT) from samples cryopreserved with egg-yolk citrate plus 7% glycerol for bovine epididymal sperm previously stored in the cauda epididymidis of testes at 4°C for 24 hours or 48 hours (h). No significant differences ($P>0.05$).

(Figure 6.5). Also, there was a significant difference found between prefreeze and 0 hour post-thaw and between prefreeze and 3-hours post-thaw mean PPM values for the cryopreserved samples that had been stored within the epididymis of testes for 48 hours ($P<0.0001$). Cryopreserved samples from the 48-hour cool storage-time treatment group also had a significant difference between the 0- and 3-hours post-thaw mean PPM values ($P=0.017$).

There was a significant difference between prefreeze and 0 hour post-thaw, as well as, between prefreeze and 3-hours post-thaw mean PIA values for the cryopreserved samples that had been stored within the epididymis for 24 hours ($P<0.0001$) (Figure 6.6). No difference was noted in the 24-hour cool storage-time treatment group between 0- and 3-hours post-thaw mean PIA values. The mean PIA values for the 48-hour cool storage-time treatment group were different between the prefreeze and 0 hour post-thaw and between the prefreeze and 3-hours post-thaw mean PIA values ($P<0.0001$).

An analysis was completed on the percent of cytoplasmic droplets that were attached to the bovine epididymal sperm and the mean CD values for prefreeze ($34.0\pm2.7\%$) and 0 hour post-thaw ($14.2\pm2.2\%$) were significantly different ($P<0.0001$).

Bovine Epididymal Sperm Parameters from Samples Cryopreserved with Milk Plus 7% Glycerol

Prefreeze PPM for bovine epididymal sperm not different between 24 and 48 hours (76.3 ± 3.5 and $64.7\pm3.5\%$, respectively) (Figure 6.7). Epididymal sperm cryopreserved in 7% MG had mean PPM values for 24 and 48 hours of cool storage-time treatment at 0 hour post-thaw of 36.9 ± 3.5 and $24.1\pm3.4\%$, respectively. At 3 hours post-thaw, mean PPM values for 24- and 48-hour cool storage-time treatment groups of 25.9 ± 3.6 and $20.6\pm3.8\%$, respectively. No difference was detected among the mean values

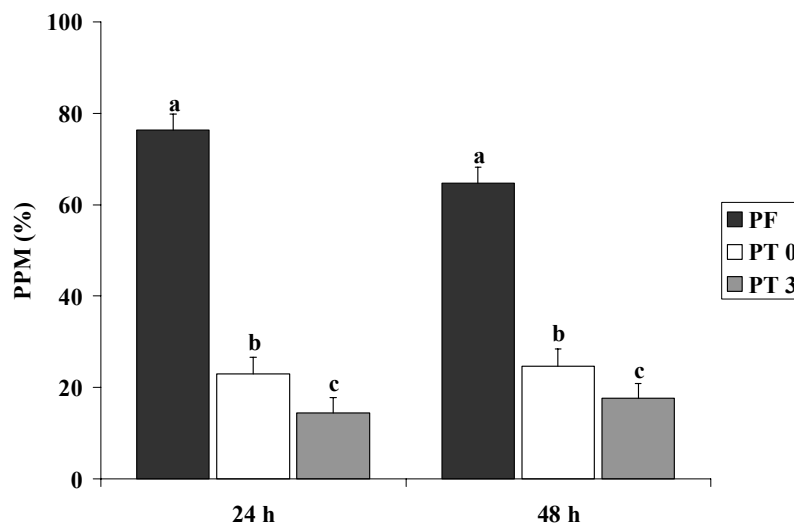


Figure 6.5. Mean (\pm SEM) percent progressive motility (PPM) at prefreeze (PF) and 0 and 3 hours post-thaw (PT) from samples cryopreserved with egg-yolk citrate plus 7% glycerol for bovine epididymal sperm previously stored in the cauda epididymidis of testes at 4°C for 24 hours or 48 hours (h). ^{a,b}Means with different superscripts within the 24-hour cool storage-time treatment group are significantly different ($P<0.0001$). ^{b,c}Means with different superscripts within the 24-hour cool storage-time treatment group are significantly different ($P=0.002$). ^{a,b} or ^cMeans with different superscripts within the 48-hour cool storage-time treatment group are significantly different ($P<0.0001$). ^{b,c}Means with different superscripts within the 48-hour cool storage-time treatment group are significantly different ($P=0.017$).

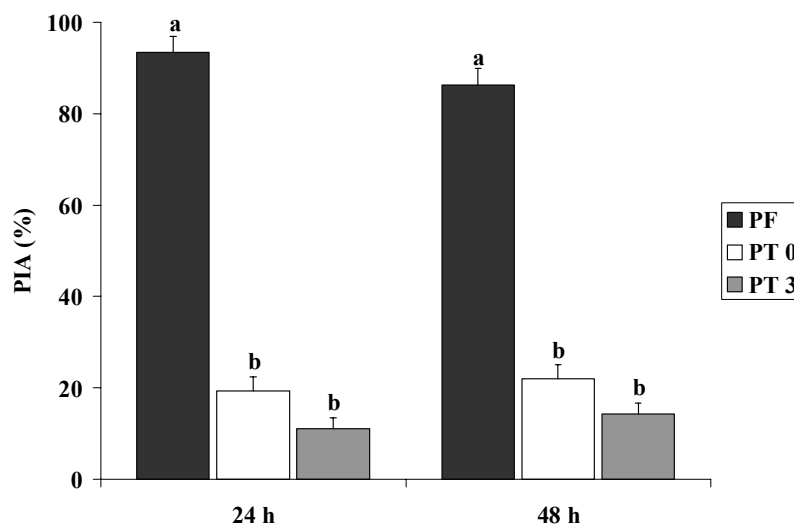


Figure 6.6. Mean (\pm SEM) percent sperm with acrosomal integrity (PIA) at prefreeze (PF) and 0 and 3 hours post-thaw (PT) from samples cryopreserved with egg-yolk citrate plus 7% glycerol for bovine epididymal sperm previously stored in the cauda epididymidis of testes at 4°C for 24 hours or 48 hours (h). ^{a,b}Means with different superscripts within storage-time treatment groups are significantly different ($P<0.0001$).

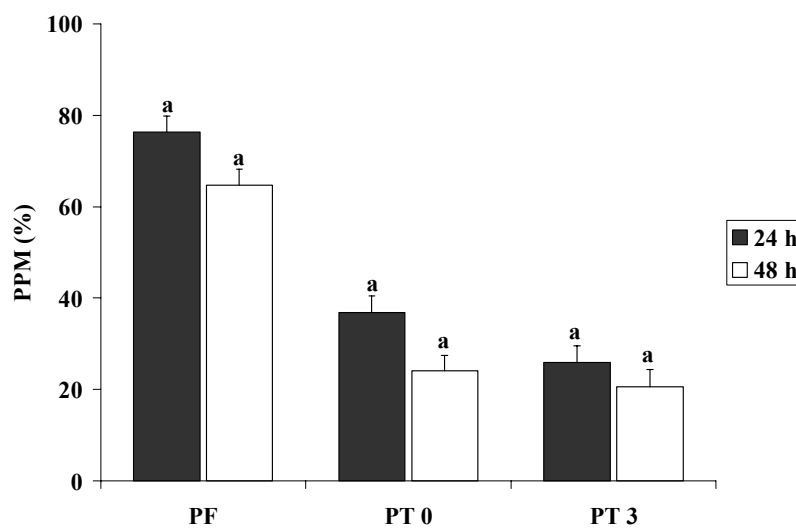


Figure 6.7. Mean (\pm SEM) percent progressive motility (PPM) at prefreeze (PF) and 0 and 3 hours post-thaw (PT) from samples cryopreserved with milk plus 7% glycerol for bovine epididymal sperm previously stored in the cauda epididymidis of testes at 4°C for 24 hours or 48 hours (h). There were no significant differences ($P>0.05$).

for PPM for either of the cool storage-time treatment groups at 0 hour or 3 hours post-thaw.

Prefreeze sperm samples had a mean PIA for 24- and 48-hours cool storage-time treatment groups of 93.3 ± 3.6 and $86.3 \pm 3.6\%$, respectively, and were not significantly different (Figure 6.8). The mean PIA values at 0 hour post-thaw for the 24- and 48-hour cool storage-time treatment groups were 41.5 ± 3.5 and $35.8 \pm 3.5\%$, respectively. Mean PIA values at 3 hours post-thaw were 30.5 ± 3.0 and $31.9 \pm 3.1\%$ for 24 and 48 hours of cool storage-time treatment groups, respectively. As with prefreeze, no difference was detected between the mean values for PIA for either of the cool storage treatment groups at either 0 hour or 3 hours post-thaw.

Sperm that had been stored at 4°C in the cauda epididymidis of testes for 24 hours and frozen in (7%MG) exhibited a significant difference between prefreeze and 0 hour post-thaw ($P=0.0433$) and prefreeze and 3 hours post-thaw ($P=0.0025$) (Figure 6.9). There was also a significant difference between 0- and 3-hour post-thaw mean PPM values for the 24-hour cool storage-time treatment group ($P=0.0399$). There was a significant difference between prefreeze and 0 hour post-thaw and prefreeze and 3 hours post-thaw for the cryopreserved samples stored within the epididymis for 48 hours ($P<0.0001$). Cryopreserved samples from the 48-hour cool storage-time treatment group were not different mean PPM values between the 0 and 3 hours post-thaw.

Mean PIA values for the cryopreserved sperm samples from the 24-hour cool storage-time treatment group were different between prefreeze and 0 hour post-thaw ($P=0.0032$) and prefreeze and 3 hour post-thaw ($P=0.0002$) (Figure 6.10). No difference was noted between the mean PIA values for the 24-hour cool storage-time treatment group at 0- and 3-hours post-thaw. A significant difference as found between the prefreeze and 0

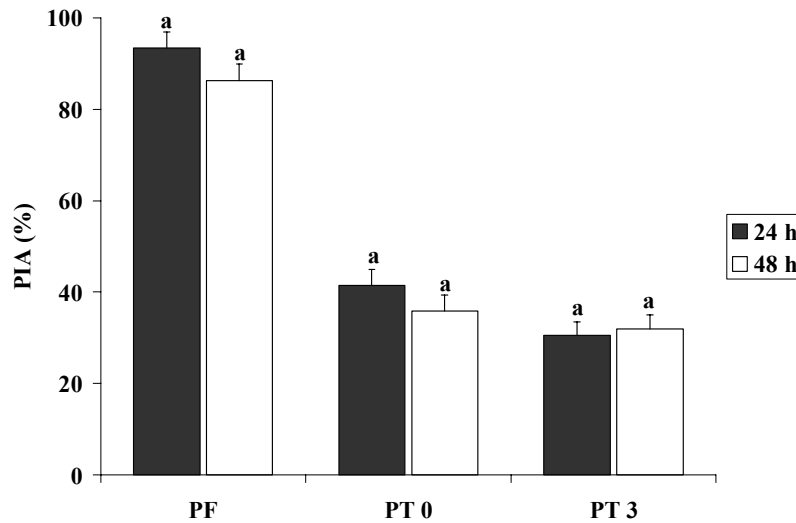


Figure 6.8. Mean (\pm SEM) percent sperm with acrosomal integrity (PIA) at prefreeze (PF) and 0 and 3 hours post-thaw (PT) from samples cryopreserved with milk plus 7% glycerol for bovine epididymal sperm previously stored in the cauda epididymidis of testes at 4°C for 24 hours or 48 hours (h). There were no significant differences ($P>0.05$).

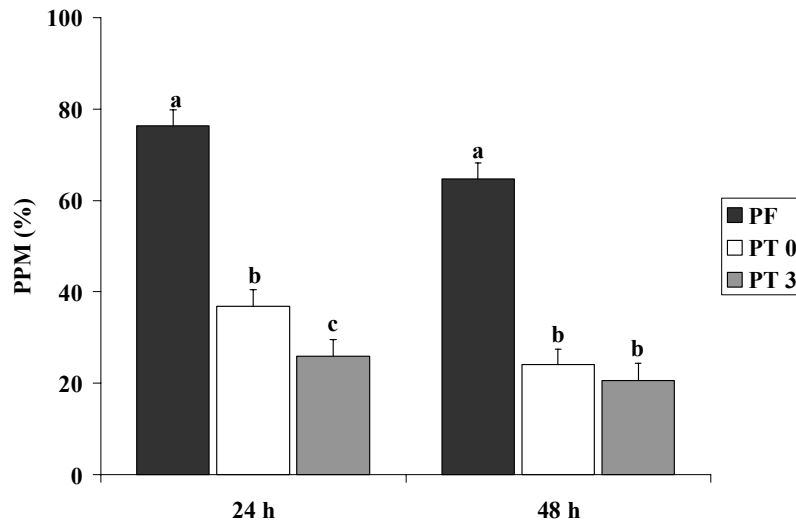


Figure 6.9. Mean (\pm SEM) percent progressive motility (PPM) at prefreeze (PF) and 0 and 3 hours post-thaw (PT) from samples cryopreserved with milk plus 7% glycerol for bovine epididymal sperm previously stored in the cauda epididymidis of testes at 4°C for 24 hours or 48 hours (h). ^{a,b}Means with different superscripts within the 24-hour cool storage-time treatment group are significantly different ($P=0.0433$). ^{a,c}Means with different superscripts within the 24-hour cool storage-time treatment group are significantly different ($P=0.0025$). ^{b,c}Means with different superscripts within the 24-hour cool storage-time treatment group are significantly different ($P=0.0399$). ^{a,b}Means with different superscripts within the 48-hour cool storage-time treatment group are significantly different ($P<0.0001$).

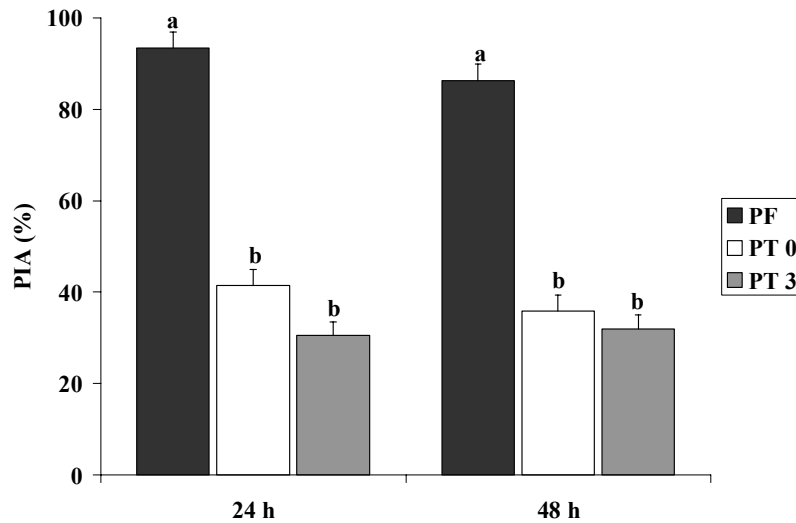


Figure 6.10. Mean (\pm SEM) percent sperm with acrosomal integrity (PIA) from samples cryopreserved with milk plus 7% glycerol for bovine epididymal sperm previously stored in the cauda epididymidis of testes at 4°C for 24 hours or 48 hours (h). ^{a,b}Means with different superscripts between prefreeze (PF) and post-thaw (PT) 0 hour in the 24-hour cool storage-time treatment group are significantly different ($P=0.0032$). ^{a,b}Means with different superscripts between prefreeze and post-thaw 3 hours in the 24-hour cool storage-time treatment group are significantly different ($P=0.0002$). ^{a,b}Means with different superscripts within the 48-hour cool storage-time treatment group are significantly different ($P<0.0001$).

hour post-thaw and between prefreeze and 3-hour post-thaw mean PIA values ($P<0.0001$) for the 48-hour cool storage-time treatment group.

Bovine Epididymal Sperm Parameters from Samples Cryopreserved with Egg-Yolk Citrate Plus 7% Glycerol Compared with Samples Preserved with Milk Plus 7% Glycerol

The mean PPM for bovine epididymal sperm exhibited no difference for the 24-hour cool storage-time treatment group between the EYG and MG treatment group or for the 48-hour cool storage-time treatment group between EYG and MG treatment groups at either 0 hour or 3 hours post-thaw (Figure 6.11).

A significant difference in mean PIA values was noted between the EYG and the MG treatment groups at 0 hour post-thaw for the 24-hour cool storage-time treatment group ($P=0.0005$) (Figure 6.12). Mean PIA values at 3 hours post-thaw for EYG and MG in both the 24- and 48-hour cool storage-time treatment groups were significantly different ($P<0.0001$ and $P=0.0007$, respectively).

The CD means for bovine epididymal sperm at prefreeze and 0 hour post-thaw are given in Figure 6.13. There was no significant difference in the percent of CD of sperm between the EYG and MG treatment groups. Mean CD values for prefreeze ($34.0\pm2.7\%$) and combined 0 hour post-thaw ($11.7\pm2.7\%$), however, were significantly different ($P<0.0001$).

Caprine Epididymal Sperm Parameters from Samples Cryopreserved with Egg-Yolk Citrate Plus 7% Glycerol

Mean PPM for caprine epididymal sperm at 24, 48, 72 and 96 hours of cool storage-time treatment groups (68.3 ± 1.6 , 55.7 ± 2.8 , 46.3 ± 2.6 and $41.9\pm2.6\%$, respectively) had a significant difference among 24 hours and 48 through 96 hours cool storage-time treatment groups ($P<0.0001$) (Figure 6.14). Cryopreserved (7% EYG) caprine epididymal sperm mean PPM values for 24, 48, 72 and 96 hours of cool storage-time treatment groups at 0 hour post-thaw were: 18.7 ± 3.4 , 20.0 ± 6.2 , 25.6 ± 5.8 and $36.3\pm5.8\%$, respectively. Post-

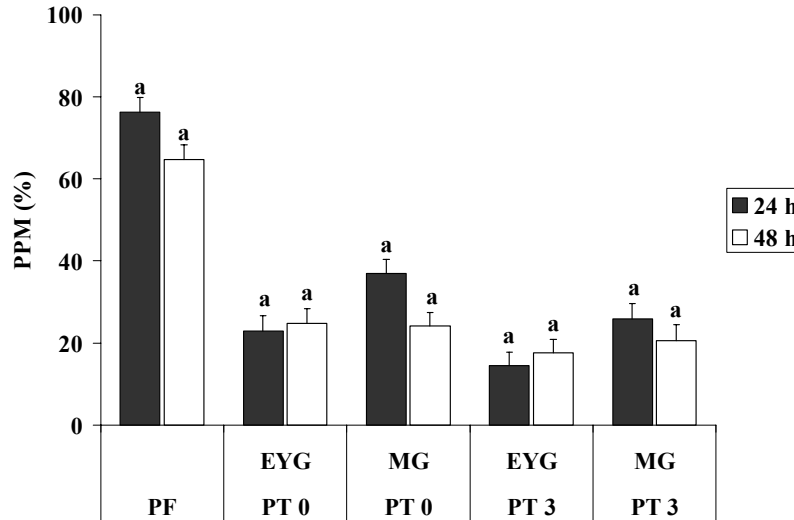


Figure 6.11. Mean (\pm SEM) percent progressively motile (PPM) sperm at prefreeze (PF), 0 and 3 hours post-thaw (PT) from samples cryopreserved with egg-yolk citrate plus 7% glycerol (EYG) or milk plus 7% glycerol (MG) diluents for bovine epididymal sperm previously stored in the cauda epididymidis of testes at 4°C for 24 hours or 48 hours (h). No significant differences ($P > 0.05$).

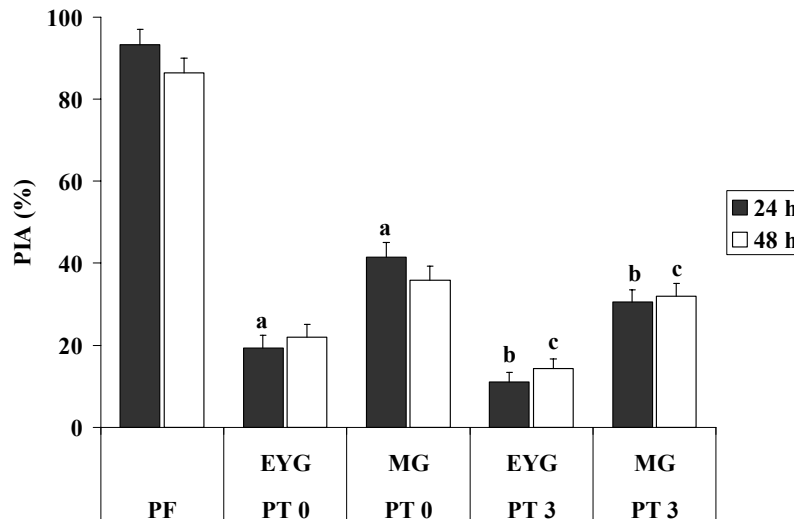


Figure 6.12. Mean (\pm SEM) percent sperm with acrosomal integrity (PIA) at prefreeze (PF), 0 and 3 hours post-thaw (PT) from samples cryopreserved with egg-yolk citrate plus 7% glycerol (EYG) or milk plus 7% glycerol (MG) for bovine epididymal sperm previously stored in the cauda epididymidis of testes at 4°C for 24 hours or 48 hours (h).

^aMeans with this superscript are significantly different from each other ($P = 0.0005$).

^bMeans with this superscript are significantly different from each other ($P < 0.0001$).

^cMeans with this superscript are significantly different from each other ($P = 0.0007$).

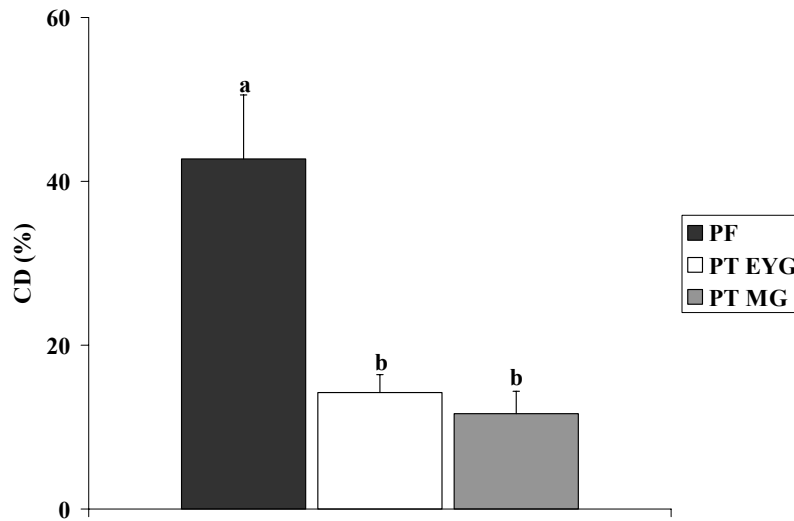


Figure 6.13. Mean (\pm SEM) percent of cytoplasmic droplets (CD) attached to bovine epididymal sperm at preefreeze (PF) and post-thaw (PT) from samples cryopreserved with egg-yolk citrate plus 7% glycerol (EYG) or milk plus 7% glycerol (MG) diluents.

^{a,b}Means with different superscripts are significantly different ($P < 0.0001$).

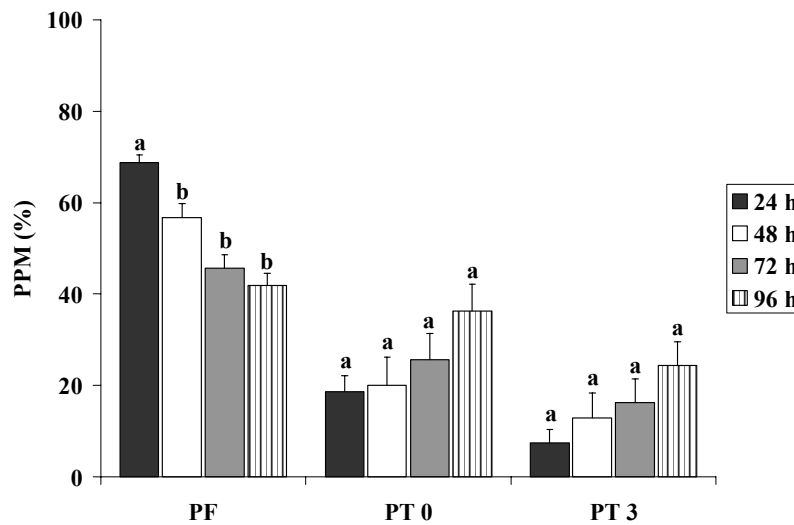


Figure 6.14. Mean (\pm SEM) percent progressive motility (PPM) at preefreeze (PF) and 0 and 3 hours post-thaw (PT) from sperm samples cryopreserved with egg-yolk citrate plus 7% glycerol for caprine epididymal sperm previously stored in the cauda epididymidis of testes at 4°C for 24, 48, 72 or 96 hours (h). ^{a,b}Means with different superscripts within time of measurement are significantly different ($P < 0.0001$).

thaw analysis after 3 hours of incubation resulted in mean PPM values for 24-, 48-, 72- and 96-hour cool storage-time treatment groups of 7.4 ± 3.0 , 12.9 ± 5.5 , 16.3 ± 5.1 and $24.4 \pm 5.1\%$, respectively. No difference was noted for PPM at any of the storage-time treatment groups for either 0 hour or 3 hours post-thaw.

An initial decrease in membrane intact sperm occurred from 24 to 48 hours while 48 through 96 hours maintained similar levels of membrane integrity throughout cool storage at 4°C with values of 66.5 ± 2.0 , 55.4 ± 3.5 , 56.8 ± 3.3 and $48.4 \pm 3.3\%$ for 24, 48, 72 and 96 hours, respectively ($P=0.0023$) (Figure 6.15). Mean PIA at 0 hour post-thaw for the 24-, 48-, 72- and 96-hour cool storage-time treatment groups were: 17.2 ± 2.4 , 20.6 ± 4.4 , 15.3 ± 4.1 and $22.6 \pm 4.1\%$, respectively. Mean PIA at 3 hours post-thaw was: 10.0 ± 1.8 , 10.6 ± 3.3 , 13.6 ± 3.1 and $13.8 \pm 3.1\%$ for 24, 48, 72 and 96 hours, respectively. As with PPM, no difference was found at 0 hour or 3 hours post-thaw for mean PIA values for any of the cool storage-time treatment groups.

Significant differences were detected in mean PPM from prefreeze to post-thaw in all cool storage-time treatment groups, except for the 96-hour cool storage-time treatment group, for samples cryopreserved with 7% EYG (Figure 6.16). Cryopreserved samples in 7% EYG after being stored in the cauda epididymidis of testes for 24 and 48 hours had a difference in mean PPM values ($P<0.0001$ and $P=0.0001$, respectively) between prefreeze and 0 hour post-thaw and between prefreeze and 3 hours post-thaw ($P<0.0001$). There was also a significant difference between prefreeze and 3-hour post-thaw mean PPM values for cryopreserved sperm samples stored within the epididymis for 72 hours ($P<0.0001$). Cryopreserved samples from the 24-hour cool storage-time treatment group were different ($P=0.0004$) between mean PPM values for 0 and 3 hours post-thaw.

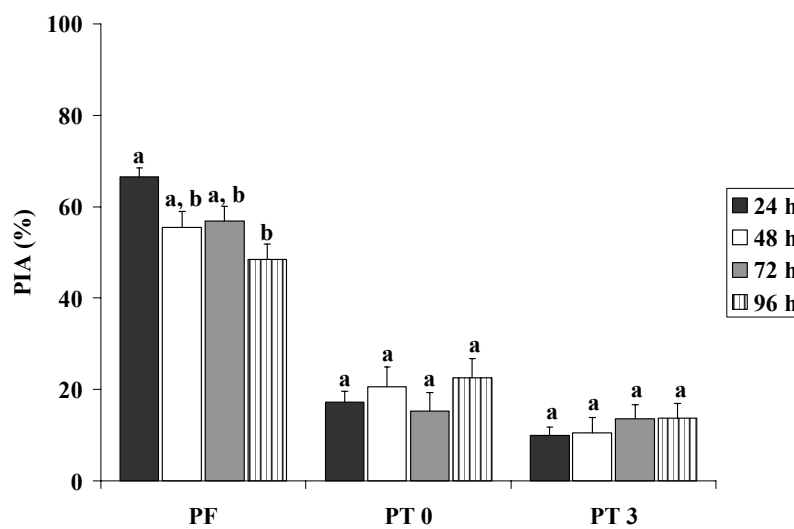


Figure 6.15. Mean (\pm SEM) percent sperm with acrosomal integrity (PIA) from samples cryopreserved with egg-yolk citrate plus 7% glycerol for caprine epididymal sperm previously stored in the cauda epididymidis of testes at 4°C for 24, 48, 72 or 96 hours (h).
^{a,b}Means with different superscripts within cool storage-time treatment groups (prefreeze, PF or post-thaw, PT) are significantly different ($P=0.0023$).

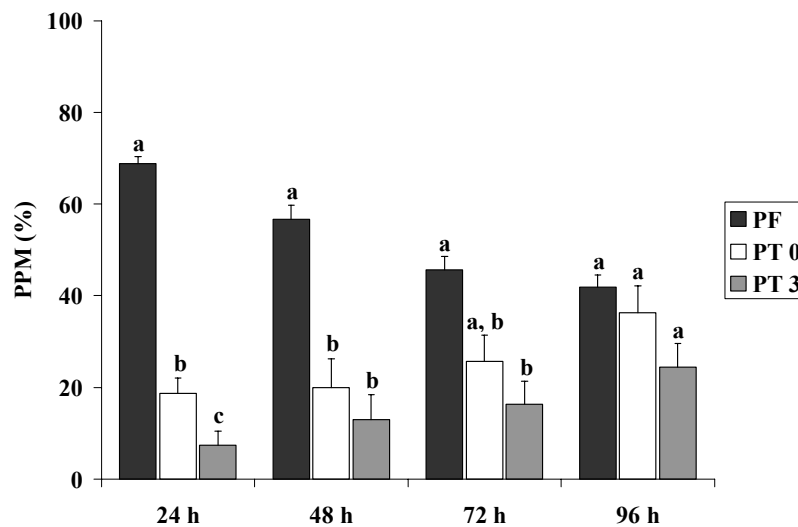


Figure 6.16. Mean (\pm SEM) percent progressive motility (PPM) from samples cryopreserved with egg-yolk citrate plus 7% glycerol for caprine epididymal sperm previously stored in the cauda epididymidis of testes at 4°C for 24, 48, 72 or 96 hours (h).
^{a,b} or ^c Means with different superscripts within the 24-hour cool storage-time treatment group are significantly different ($P < 0.0001$). ^{b,c} Means with different superscripts within the 24-hour cool storage-time treatment group are significantly different ($P = 0.0004$).
^{a,b} Means with different superscripts between prefreeze (PF) and 0 hour post-thaw (PT) in the 48-hour cool storage-time treatment group are significantly different ($P = 0.0001$) and between prefreeze and 3 hours post-thaw in the 48-hour cool storage-time treatment group are significantly different ($P < 0.0001$). ^{a,b} Means with different superscripts within the 72-hour cool storage-time treatment group are significantly different ($P < 0.0001$).

Prefreeze and 0-hour post-thaw mean PIA values for cryopreserved (7% EYG) samples from 24, 48, 72 and 96 hours of cool storage-time treatment groups exhibited a difference ($P<0.0001$ for 24- through 72-hour groups and $P=0.0004$ for 96-hour group) (Figure 6.17). All cool storage-time treatment groups (24 to 96 hours) were significantly different ($P<0.0001$) in mean PIA values between prefreeze and 3 hours post-thaw.

Caprine Epididymal Sperm Parameters from Samples Cryopreserved with Milk Plus 7% Glycerol

Mean PPM for caprine epididymal sperm at 24, 48, 72 and 96 hours of cool storage-time treatment groups (68.3 ± 1.6 , 55.7 ± 2.8 , 46.3 ± 2.6 and $41.9\pm2.6\%$, respectively) exhibited a decrease from 24 hours to 48 through 96 hours of cool storage-time treatment groups ($P<0.0001$) (Figure 6.18). Caprine epididymal sperm cryopreserved in 7% MG had mean PPM values for 24, 48, 72 and 96 hours of cool storage-time treatment groups at 0 hour post-thaw of 39.5 ± 3.6 , 42.5 ± 6.6 , 37.1 ± 6.2 and $31.3\pm5.8\%$, respectively.

Mean PPM values at 3 hour post-thaw for 24-, 48-, 72- and 96-hours cool storage-time treatment groups of were: 31.4 ± 3.2 , 35.8 ± 5.9 , 34.3 ± 5.5 and $30.0\pm5.1\%$, respectively. No difference was noted among mean PPM for any of the cool storage-time treatment groups at either 0 hour or 3 hours post-thaw.

Sperm PIA resulted in an initial decrease from 24 to 48 hours with 48 through 96 hours of cool storage-time treatment groups maintaining membrane integrity throughout storage at 4°C (66.5 ± 2.0 , 55.4 ± 3.5 , 56.8 ± 3.3 and $48.4\pm3.3\%$ for 24, 48, 72 and 96 hours, respectively) ($P=0.0023$) (Figure 6.19). The mean PIA values at 0 hour post-thaw for the 24-, 48-, 72- and 96-hour cool storage-time treatment groups were: 34.6 ± 2.6 , 28.7 ± 4.8 ,

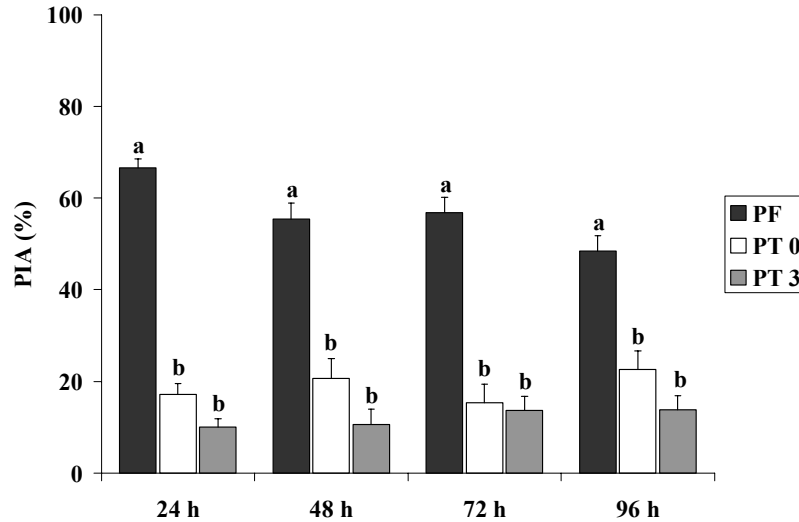


Figure 6.17. Mean (\pm SEM) percent sperm with membrane integrity (PIA) from samples cryopreserved with egg-yolk citrate plus 7% glycerol for caprine epididymal sperm previously stored in the cauda epididymidis of testes at 4°C for 24, 48, 72 or 96 hours (h). ^{a,b}Means with different superscripts between preefreeze (PF) and 0 hour post-thaw (PT) in the 24-, 48- and 72-hour cool storage-time treatment groups are significantly different ($P<0.0001$) and between preefreeze and 0 hour post-thaw in the 96-hour cool storage-time treatment group are significantly different ($P=0.0004$). ^{a,b}Means with different superscripts between preefreeze and 3 hours post-thaw within the cool storage-time treatment groups are significantly different ($P<0.0001$).

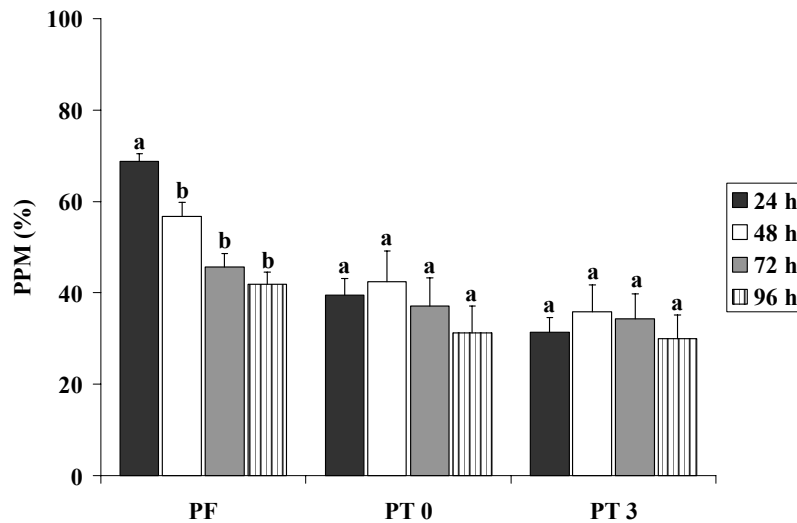


Figure 6.18. Mean (\pm SEM) percent progressive motility (PPM) at preefreeze (PF) and 0 and 3 hours post-thaw (PT) from samples cryopreserved with milk plus 7% glycerol for caprine epididymal sperm previously stored in the cauda epididymidis of testes at 4°C for 24, 48, 72 or 96 hours (h). ^{a,b}Means with different superscripts within storage-time treatment groups are significantly different ($P<0.0001$).

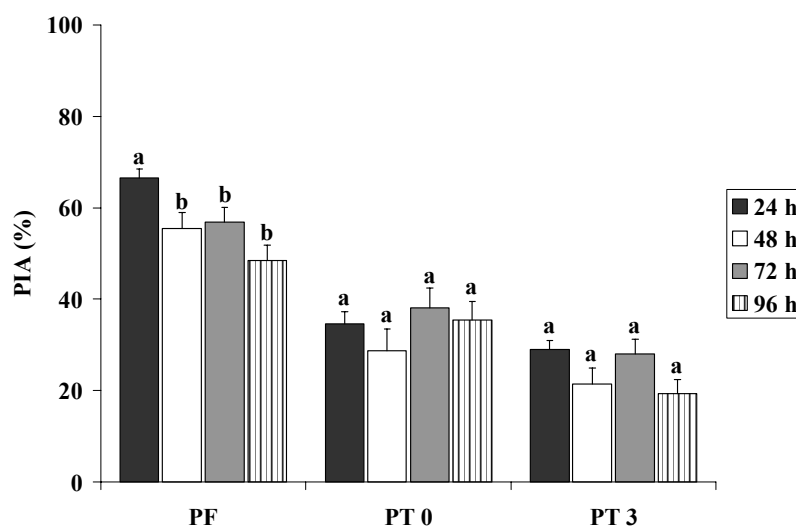


Figure 6.19. Mean (\pm SEM) percent sperm with acrosomal integrity (PIA) from samples cryopreserved with milk plus 7% glycerol for caprine epididymal sperm previously stored in the cauda epididymidis of testes at 4°C for 24, 48, 72 or 96 hours (h). ^{a,b}Means with different superscripts within time of measurement (prefreeze, PF or post-thaw, PT) are significantly different ($P=0.0023$).

38.0±4.4 and 35.4±4.1%, respectively. Mean PIA values at 3 hours post-thaw were: 29.0±1.9, 21.4±3.6, 28.0±3.3 and 19.3±3.1% for the groups cooled for 24, 48, 72 and 96 hours, respectively. No difference was noted among mean values for PIA for any of the cool storage-time treatment groups at 0 hour or 3 hours post-thaw.

Cryopreserved sperm samples in 7%MG after being stored in the cauda epididymidis of testes for 24 hours of cool storage-time treatment had a difference ($P<0.0001$) between prefreeze and 0-hour post-thaw as well as between prefreeze and 3-hour post-thaw mean PPM values ($P<0.0001$) (Figure 6.20).

Mean PIA values for cryopreserved samples from the 24- and 48-hour cool storage-time treatment groups exhibited differences between prefreeze and 0 hour post-thaw ($P<0.0001$ and $P=0.0077$, respectively) (Figure 6.21). All three cool storage-time treatment groups (24 to 72 hours) were significantly different ($P<0.0001$) for mean PIA values between prefreeze and 3-hour post-thaw. A significant difference was also found in the 96-hour cool storage-time treatment group between the 0- and 3-hour post-thaw mean PIA values ($P=0.0124$).

Caprine Epididymal Sperm Parameters from Samples Cryopreserved with Egg-Yolk Citrate plus 7% Glycerol Compared with Samples Preserved with Milk Plus 7% Glycerol

Mean PPM for the 24-hour cool storage-time treatment group at 0 hour and at 3 hours post-thaw was significantly different between samples cryopreserved in EYG compared with MG media ($P=0.0115$ and $P=0.0001$, respectively) (Figure 6.22). In both 0 hour and 3 hours post-thaw for the 24-hour cool storage-time treatment group, the MG medium sustained greater progressive motility than in the EYG medium. No differences were found for mean PPM among samples cryopreserved in EYG compared with MG for the rest of the cool storage-time treatment groups (48, 72 and 96 hours).

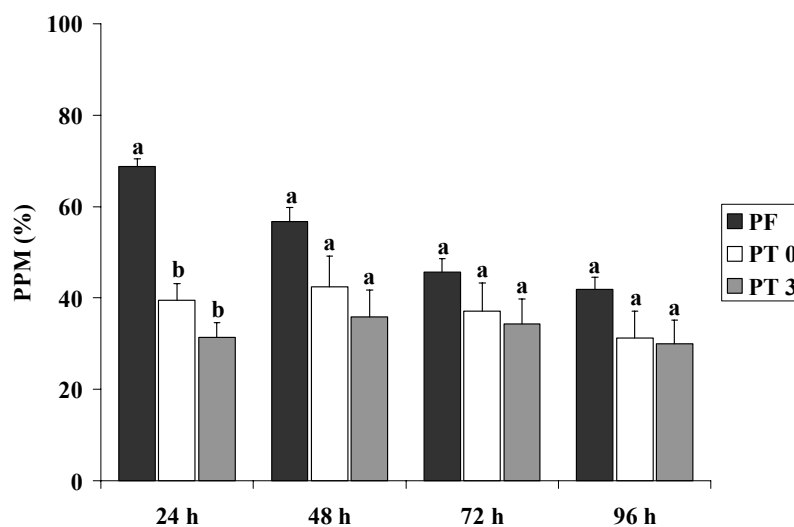


Figure 6.20. Mean (\pm SEM) percent progressive motility (PPM) from samples cryopreserved with milk plus 7% glycerol for caprine epididymal sperm previously stored in the cauda epididymidis of testes at 4°C for 24, 48, 72 or 96 hours (h). ^{a,b}Means with different superscripts within storage-time treatment groups (prefreeze, PF or post-thaw, PT) are significantly different ($P < 0.0001$).

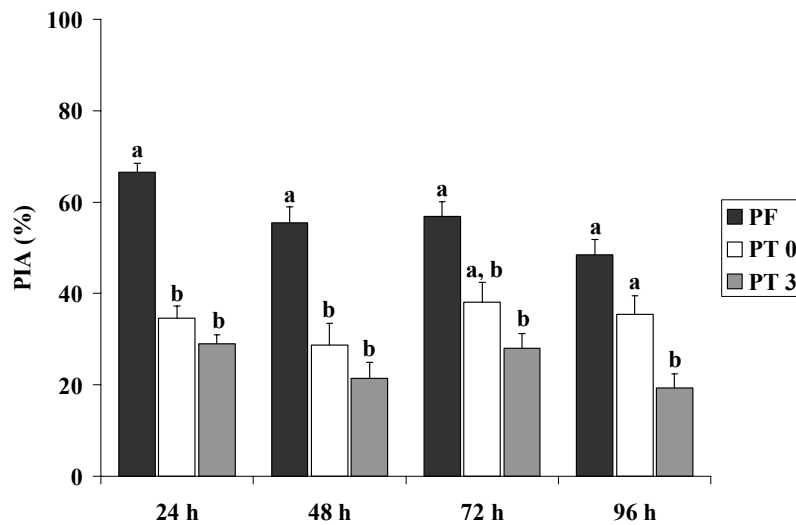


Figure 6.21. Mean (\pm SEM) percent sperm with intact acrosomal membranes (PIA) from samples cryopreserved with milk plus 7% glycerol for caprine epididymal sperm previously stored in the cauda epididymidis of testes at 4°C for 24, 48, 72 or 96 hours (h). ^{a,b}Means with different superscripts within the 24-hour cool storage-time treatment group are significantly different ($P < 0.0001$). ^{a,b}Means with different superscripts between prefreeze (PF) and 0 hour post-thaw (PT) in the 48-hour cool storage-time treatment group are significantly different ($P = 0.0077$) and between prefreeze and 3 hours post-thaw in the 48-hour cool storage-time treatment group are significantly different ($P < 0.0001$). ^{a,b}Means with different superscripts within the 72-hour cool storage-time treatment group are significantly different ($P < 0.0001$). ^{a,b}Means with different superscripts within the 96-hour cool storage-time treatment group are significantly different ($P = 0.0124$).

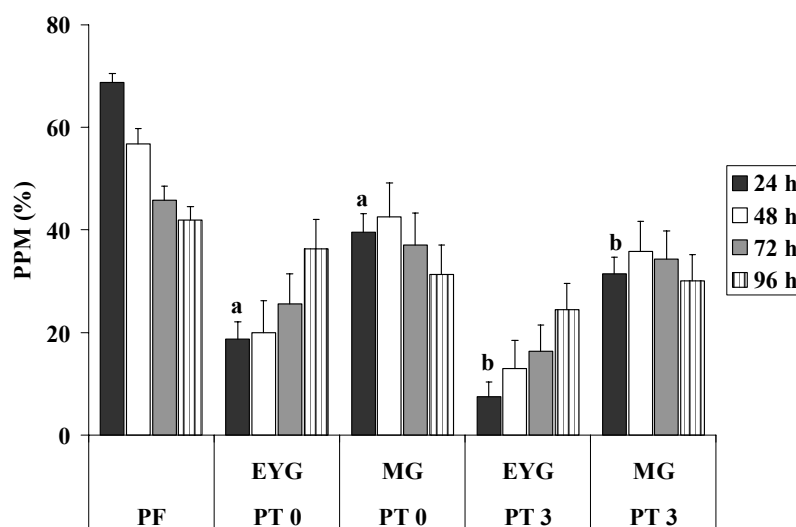


Figure 6.22. Mean (\pm SEM) percent progressively motile (PPM) sperm at preeeze (PF), 0 and 3 hours post-thaw (PT) from samples cryopreserved with egg-yolk citrate plus 7% glycerol (EYG) or milk plus 7% glycerol (MG) diluents for caprine epididymal sperm previously stored in the cauda epididymidis of testes at 4°C for 24 hours or 48 hours (h). ^aMeans with this superscript are significantly different from other columns with the same superscript (P=0.0115). ^bMeans with this superscript are significantly different from other columns with the same superscript (P=0.0001).

The differences noted for mean PPM between EYG and MG cryoprotectant were also detected among the 24-hour cool storage-time treatment group at 0 hour and 3 hours post-thaw ($P=0.0011$ and $P<0.0001$, respectively) for mean PIA (Figure 6.23). No differences were found for mean PIA between the EYG cryoprotectant compared with the MG cryoprotectant for the remaining cool storage-time treatment groups (48, 72 and 96 hours) and measurement times (0 hour and 3 hours).

Equine Epididymal Sperm Parameters from Samples Cryopreserved with Skim Milk Egg Yolk Plus 4% Glycerol

Equine epididymal sperm mean PPM value exhibited a downward trend from 24 to 96 hours of cool storage-time treatment groups (57.0 ± 3.3 , 41.7 ± 5.2 , 39.0 ± 5.7 and $35.0\pm6.3\%$, respectively) (Figure 6.24). Sperm cryopreserved in 4% SM-EYG had mean PPM values for 24, 48, 72 and 96 hours of cool storage-time treatment groups at 0 hour post-thaw of 46.4 ± 2.4 , 38.4 ± 3.8 , 47.0 ± 4.2 and $42.5\pm4.7\%$, respectively. At 3 hours post-thaw, mean PPM values for 24-, 48-, 72- and 96-hour cool storage-time treatment groups of 30.7 ± 3.3 , 23.4 ± 5.2 , 30.0 ± 5.7 and $28.8\pm6.4\%$, respectively. No difference was detected for mean PPM for any of the cool storage-time treatment groups at any of the measurement times.

Acrosomal integrity was maintained from sperm samples in all storage groups as indicated by mean PIA for 24, 48, 72 and 96 hours of cool storage-time treatment groups (76.7 ± 2.1 , 71.4 ± 3.3 , 71.4 ± 3.6 and $66.8\pm4.1\%$, respectively) (Figure 6.25). Mean PIA values at 0 hour post-thaw for the 24-, 48-, 72- and 96-hour cool storage-time treatment groups were: 32.4 ± 3.0 , 34.2 ± 4.8 , 34.4 ± 5.2 and $17.5\pm4.4\%$, respectively. Mean PIA values at 3 hours post-thaw were 16.4 ± 2.3 , 21.4 ± 3.6 , 19.2 ± 3.9 and $35.0\pm4.4\%$ for 24, 48, 72 and 96 hours of cool storage-time treatment groups, respectively. No differences were found

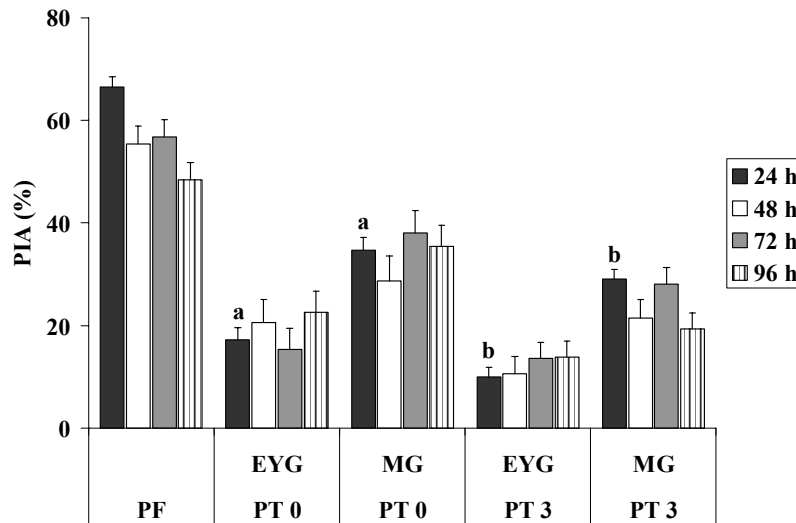


Figure 6.23. Mean (\pm SEM) percent sperm with acrosomal integrity (PIA) at prefreeze (PF), 0 and 3 hours post-thaw (PT) from samples cryopreserved with egg-yolk citrate plus 7% glycerol (EYG) or milk plus 7% glycerol (MG) diluents for caprine epididymal sperm previously stored in the cauda epididymidis of testes at 4°C for 24 hours or 48 hours (h). ^aMeans with this superscript are significantly different from other columns with the same superscript ($P=0.0011$). ^bMeans with this superscript are significantly different from other columns with the same superscript ($P<0.0001$).

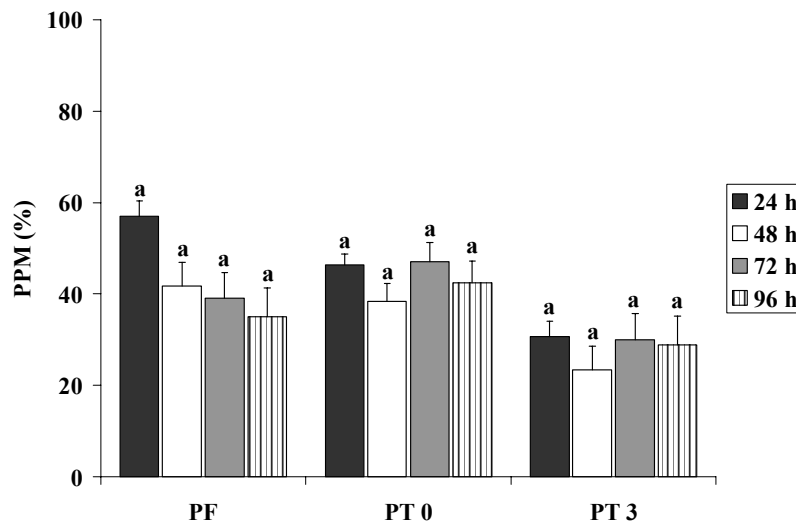


Figure 6.24. Mean (\pm SEM) percent progressive motility (PPM) at prefreeze (PF) and 0 and 3 hours post-thaw (PT) from samples cryopreserved with skim milk egg yolk plus 4% glycerol for equine epididymal sperm previously stored in the cauda epididymidis of testes at 4°C for 24, 48, 72 or 96 hours (h). There was no significant differences ($P>0.05$).

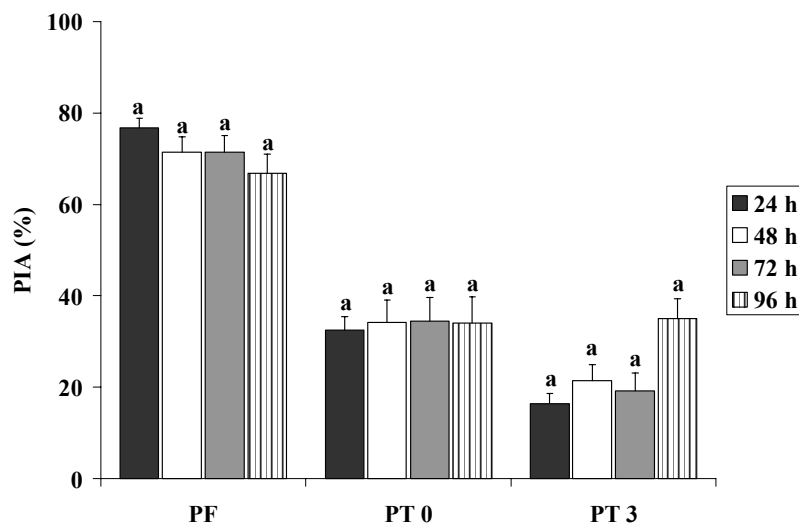


Figure 6.25. Mean (\pm SEM) percent sperm with acrosomal integrity (PIA) at prefreeze (PF) and 0 and 3 hours post-thaw (PT) from samples cryopreserved with skim milk egg yolk plus 4% glycerol for equine epididymal sperm previously stored in the cauda epididymidis of testes at 4°C for 24, 48, 72 or 96 hours (h). There was no significant differences ($P>0.05$).

among the mean PIA values for any of the storage groups at any of the measurement times.

Sperm that had been stored at 4°C in the cauda epididymis of testes for 24, 48 and 72 hours and frozen in 4% SM-EYG were found to have a difference ($P<0.0001$) between prefreeze and 3 hours post-thaw and between 0 hour and 3 hours post-thaw for mean PPM values ($P<0.0001$, $P=0.0366$ and $P=0.0249$ for 24, 48 and 72 hours, respectively) (Figure 6.26).

Prefreeze and 0-hour post-thaw mean PIA values for the cryopreserved sperm samples of all cool storage-time treatment groups were significantly different ($P<0.0001$ for 24 and 48 hours, $P=0.0005$ for 72 and 96 hours) (Figure 6.27). Only the 24-hour cool storage-time treatment group resulted in a significant difference ($P<0.0001$) between prefreeze and 3-hour post-thaw mean PIA. Furthermore, the mean CD values for prefreeze ($30.3\pm2.3\%$) and 0 hour post-thaw ($7.8\pm1.9\%$) were significantly different ($P<0.0001$).

Equine Epididymal Sperm Parameters from Samples Cryopreserved with Egg-Yolk Citrate Plus 4% Glycerol

Mean PPM values for prefreeze equine epididymal sperm displayed a downward trend from 24 to 96 hours of cool storage-time treatment groups (57.0 ± 3.3 , 41.7 ± 5.2 , 39.0 ± 5.7 and $35.0\pm6.3\%$, respectively) (Figure 6.28). Sperm cryopreserved in 4% EYG after 24, 48, 72 and 96 hours of cool storage-time treatment groups, assessed at 0 hour post-thaw had mean PPM values of 20.0 ± 2.4 , 12.0 ± 3.8 , 18.0 ± 4.2 and $21.0\pm4.7\%$, respectively. Analysis at 3 hours post-thaw produced mean PPM values for 24-, 48-, 72- and 96-hour cool storage-time treatment groups of 10.0 ± 3.3 , 3.0 ± 5.2 , 3.0 ± 5.7 and $2.5\pm6.4\%$, respectively. No difference was detected among the mean PPM values for any of the cool storage-time treatment groups.

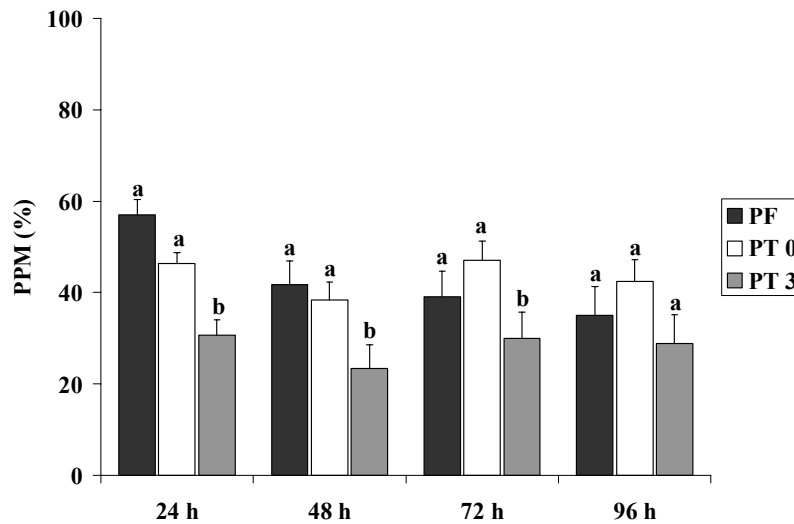


Figure 6.26. Mean (\pm SEM) percent progressive motility (PPM) from samples cryopreserved with skim milk egg yolk plus 4% glycerol for equine epididymal sperm previously stored in the cauda epididymidis of testes at 4°C for 24, 48, 72 or 96 hours (h). ^{a,b}Means with different superscripts between prefreeze (PF) and 3 hours post-thaw (PT) in the cool storage-time treatment groups are significantly different ($P < 0.0001$). ^{a,b}Means with different superscripts between 0 hour and 3 hours post-thaw in the 24-hour cool storage-time treatment group are significantly different ($P < 0.0001$) and in the 48-hour cool storage-time treatment group are significantly different ($P = 0.0366$) and in the 72-hour cool storage-time treatment group are significantly different ($P = 0.0249$).

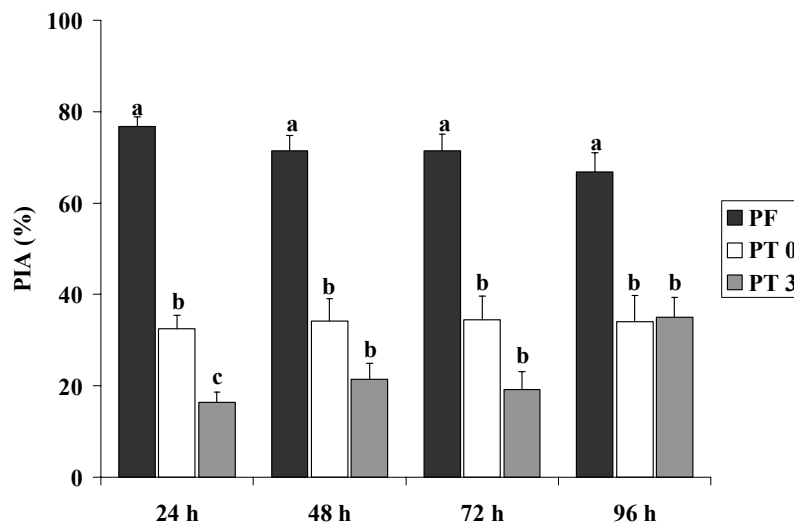


Figure 6.27. Mean (\pm SEM) percent sperm with acrosomal integrity (PIA) from samples cryopreserved with skim milk egg yolk plus 4% glycerol for equine epididymal sperm previously stored in the cauda epididymidis of testes at 4°C for 24, 48, 72 or 96 hours (h). ^{a,b,c}Means with different superscripts within the 24-hour cool storage-time treatment group (prefreeze, PF or post-thaw, PT) are significantly different ($P < 0.0001$). ^{a,b}Means with different superscripts within the 48-hour cool storage-time treatment group are significantly different ($P < 0.0001$). ^{a,b}Means with different superscripts within the 72- and 96-hour cool storage-time treatment group are significantly different ($P = 0.0005$).

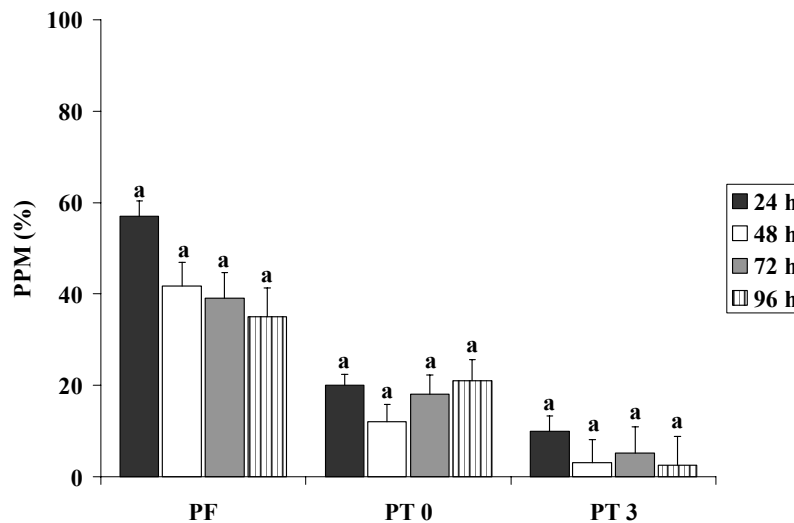


Figure 6.28. Mean (\pm SEM) percent progressive motility (PPM) at prefreeze (PF) and 0 and 3 hours post-thaw (PT) from samples cryopreserved with egg-yolk citrate plus 4% glycerol for equine epididymal sperm previously stored in the cauda epididymidis of testes at 4°C for 24, 48, 72 or 96 hours (h). There was no significant differences ($P > 0.05$).

Prefreeze mean PIA values of equine epididymal sperm for 24, 48, 72 and 96 hours of cool storage-time treatment groups was 76.7 ± 2.1 , 71.4 ± 3.3 , 71.4 ± 3.6 and $66.8 \pm 4.1\%$, respectively (Figure 6.29). The mean PIA values at 0 hour post-thaw for the 24-, 48-, 72- and 96-hour cool storage-time treatment groups were: 21.8 ± 3.0 , 14.0 ± 4.8 , 24.6 ± 5.2 and $27.0 \pm 5.8\%$, respectively. Sperm PIA at 3 hours post-thaw were: 15.0 ± 2.3 , 18.7 ± 3.6 , 21.2 ± 3.9 and $17.5 \pm 4.4\%$ for 24, 48, 72 and 96 hours of cool storage-time treatment groups, respectively. No significant difference was detected among the mean values for PIA for any of the cool storage-time treatment groups for any time measurements.

Cryopreserved epididymal sperm (4% EYG) from previously stored in the cauda epididymidis for 24, 48, 72 and 96 hours of cool storage-time treatment groups exhibited a significant difference ($P < 0.0001$ for the 24- through the 72-hour treatments and $P = 0.0164$ for the 96-hour treatment) between prefreeze and 0 hour post-thaw and ($P < 0.0001$ for the 24- through the 72-hour treatments and $P = 0.0415$ for the 96-hour treatment) between prefreeze and 3-hour post-thaw mean PPM values (Figure 6.30).

A significant difference between prefreeze and 0-hour post-thaw mean PIA values for equine epididymal sperm cryopreserved samples (4% EYG) was found of all cool storage-time treatment groups ($P < 0.0001$ for the 24- through the 72-hour treatments and $P = 0.0009$ for the 96-hour treatment) (Figure 6.31). All storage groups were also significantly different between prefreeze and 3 hours post-thaw for mean PIA values ($P < 0.0001$).

In addition, mean CD values for prefreeze ($30.3 \pm 2.3\%$) and 0 hour post-thaw ($5.5 \pm 1.9\%$) assessments of equine epididymal sperm were significantly different ($P < 0.0001$).

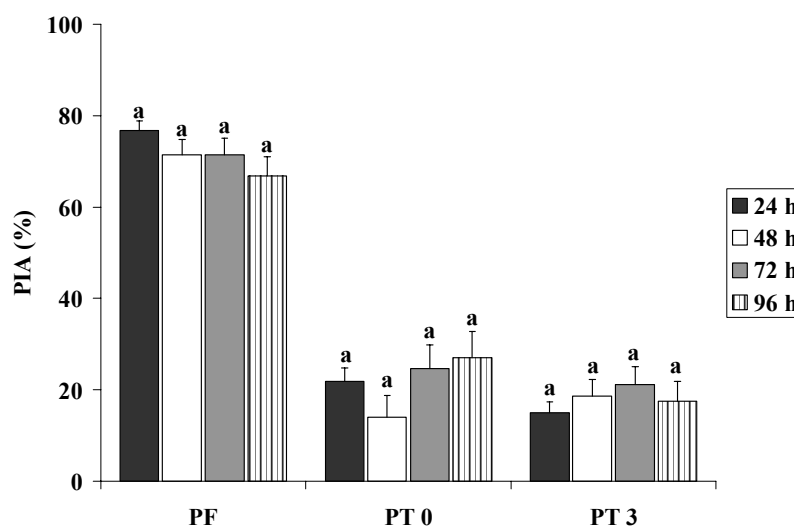


Figure 6.29. Mean (\pm SEM) percent sperm with acrosomal integrity (PIA) at preefreeze (PF) and 0 and 3 hours post-thaw (PT) from samples cryopreserved with egg yolk plus 4% glycerol for equine epididymal sperm previously stored in the cauda epididymidis of testes at 4°C for 24, 48, 72 or 96 hours (h). There was no significant differences ($P > 0.05$).

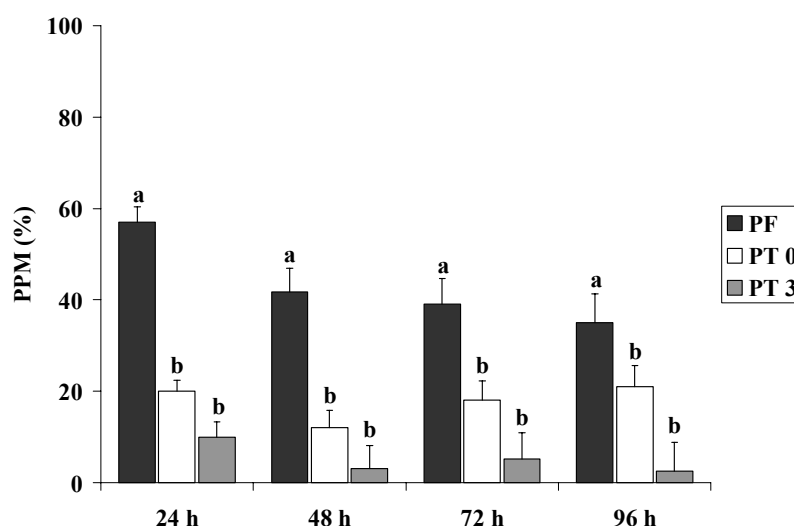


Figure 6.30. Mean (\pm SEM) percent progressive motility (PPM) from samples cryopreserved with egg yolk plus 4% glycerol for equine epididymal sperm previously stored in the cauda epididymidis of testes at 4°C for 24, 48, 72 or 96 hours (h). ^{a,b}Means with different superscripts within the 24-, 48- and 72-hour cool storage-time treatment groups are significantly different ($P < 0.0001$). ^{a,b}Means with different superscripts between preefreeze (PF) and 0 hour post-thaw (PT) in the 96-hour cool storage-time treatment group are significantly different ($P = 0.0164$) and between preefreeze and 3 hours post-thaw in the 96-hour cool storage-time treatment group are significantly different (0.0415).

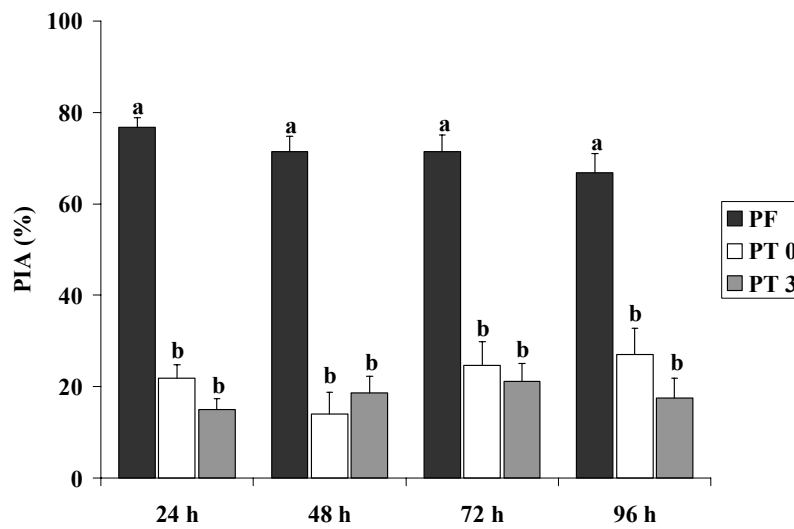


Figure 6.31. Mean (\pm SEM) percent sperm with acrosomal integrity (PIA) from samples cryopreserved with egg yolk plus 4% glycerol for equine epididymal sperm previously stored in the cauda epididymidis of testes at 4°C for 24, 48, 72 or 96 hours (h). ^{a,b}Means with different superscripts within the 24-, 48- and 72-hour cool storage-time treatment groups are significantly different ($P < 0.0001$). ^{a,b}Means with different superscripts between prefreeze (PF) and 0 hour post-thaw (PT) in the 96-hour cool storage-time treatment groups are significantly different ($P = 0.0009$) and between prefreeze and 3 hours post-thaw in the 96-hour cool storage-time treatment groups are significantly different ($P < 0.0001$).

Equine Epididymal Sperm Parameters from Samples Cryopreserved with Skim Milk Egg Yolk Plus 4% Glycerol Compared with Samples Preserved with Egg-Yolk Citrate Plus 4% Glycerol

A significant difference for all mean PPM of all cool storage-time treatment groups (24, 48, 72 and 96 hours) at 0 hour post-thaw was found between SM-EYG and EYG cryoprotectants ($P < 0.0001$) (Figure 6.32). Equine epididymal sperm cryopreserved in SM-EYG medium maintained greater mean PPM than sperm samples preserved in EYG diluent. No significant difference was found for PIA between the two cryoprotectant media (SM- EYG compared with EYG) at 0 hour or 3 hours post-thaw for any of the four cool storage-time treatment groups (Figure 6.33). Furthermore, there was a difference in mean CD values attached from prefreeze to 0 hour post-thaw in both the 24- and 48-hour cool storage-time treatment groups ($P < 0.0001$) (Figure 6.34). There was no difference between SM-EYG and EYG cryoprotectants in the percent CD retained on the post-thaw sperm in either of the cool storage-time treatment groups.

Discussion

Analysis of prefreeze PPM and PIA differences among the initial cool storage-time treatment groups of 24, 48, 72 and 96 hours of storage in the testicle at 4°C show similar decreasing values as previously reported by the author (James et al., 2002; Chapters 3, 4 and 5) for the bovine, caprine and equine males. There was a difference as to when the decline in mean values occurred for PPM and PIA related to the species (bovine, caprine or equine) evaluated in this study.

Samples of bovine epididymal sperm stored in the cauda epididymidis of testes for 24 and 48 hours postmortem at 4°C and cryopreserved in EYG or MG had a significant decrease in mean PPM over time from prefreeze to 0 and to 3 hours post-thaw. The initial

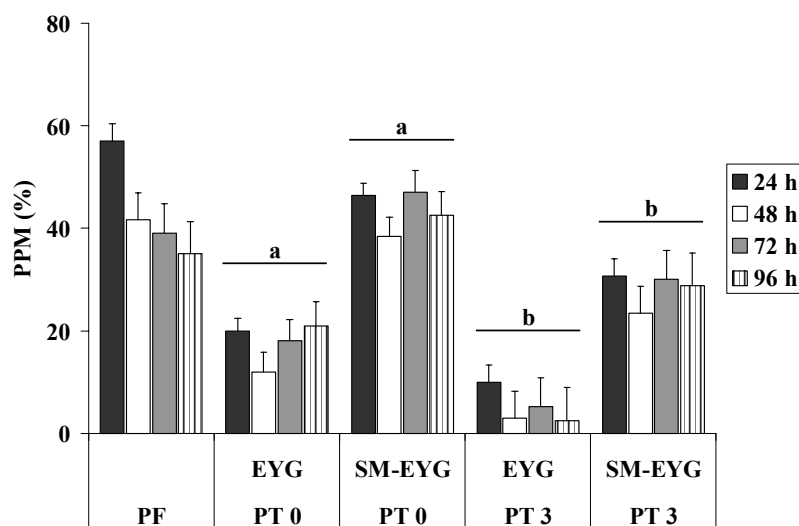


Figure 6.32. Mean (\pm SEM) percent progressively motile (PPM) sperm at prefreeze (PF), 0 and 3 hours post-thaw (PT) from samples cryopreserved with egg-yolk citrate plus 4% glycerol (EYG) or skim milk egg yolk plus 4% glycerol (SM-EYG) for equine epididymal sperm previously stored in the cauda epididymidis of testes at 4°C for 24, 48, 72 or 96 hours (h). ^{a,b}Medium treatment groups with the same superscript are significantly different ($P<0.0001$).

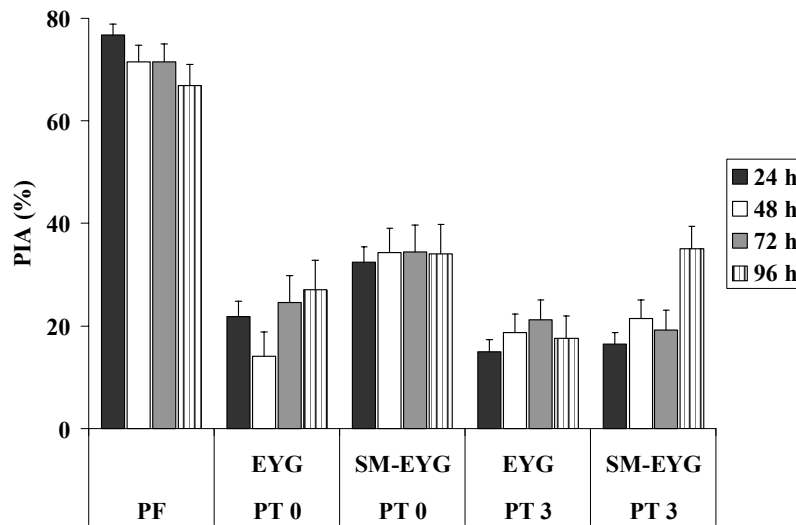


Figure 6.33. Mean (\pm SEM) percent sperm with acrosomal integrity (PIA) at preefreeze (PF), 0 and 3 hours post-thaw (PT) from samples cryopreserved with egg-yolk citrate plus 4% glycerol (EYG) or skim milk egg yolk plus 4% glycerol (SM-EYG) for equine epididymal sperm previously stored in the cauda epididymidis of testes at 4°C for 24, 48, 72 or 96 hours (h). There was no significant differences ($P>0.05$).

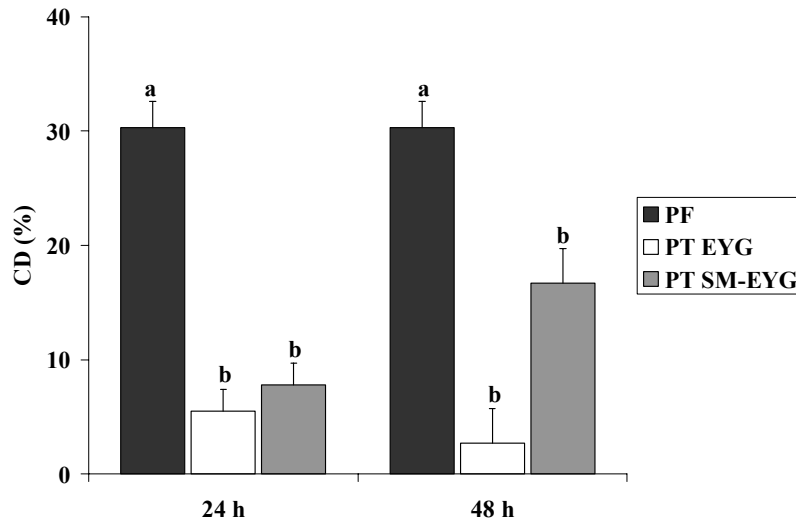


Figure 6.34. Mean (\pm SEM) percent of cytoplasmic droplets (CD) attached to sperm at preefreeze (PF) and post-thaw (PT) from samples cryopreserved with egg-yolk citrate plus 4% glycerol (EYG) or skim milk egg yolk plus 4% glycerol (SM-EYG) for equine epididymal sperm. ^{a,b}Means with different superscripts within cool storage-time treatment groups (h) are significantly different ($P<0.0001$).

decrease in PPM from prefreeze to 0 hour post-thaw is indicative of damage from the cryopreservation process, an immediate outcome. The decrease in PPM at 3 hours post-thaw was from latent cryoinjury that required a longer incubation time to become apparent. The initial decrease noted from prefreeze to 0 hour post-thaw for PPM in bovine epididymal sperm was greater than the PPM decline reported by Kilian et al. (2000) for cryopreserved epididymal sperm of the African buffalo. This decrease in PPM of domestic bovine epididymal sperm is in contrast to the increase in PPM from prefreeze to post-thaw reported for the epididymal gaur sperm (Hopkins et al., 1988).

The latent decrease in PPM at 3 hours has been correlated with decreased fertility with ejaculated sperm for those bulls displaying this trait (Saacke, 1970; Fleming et al., 1976; Chandler et al., 1978). Mean PIA values only exhibited a decrease from prefreeze to 0 hour post-thaw. It should not be overlooked that frozen-thawed bovine epididymal sperm has been used to produce offspring by using the ICSI procedure (Goto et al., 1990; Goto, 1993).

In the present study, there was a ~50% loss of the retained cytoplasmic droplets on the bovine epididymal sperm from prefreeze to 0 hour post-thaw. Loss of attached cytoplasmic droplets from sperm after the freeze-thaw process is most likely due to osmotic differences created by the addition of glycerol, freezing and/or thawing (Hopkins et al., 1988). Evaluating the loss of cytoplasmic droplets could be a means of assessing the extent of cryodamage to epididymal sperm. Ejaculated sperm with retained cytoplasmic droplets of >30% has been correlated to lowered IVF fertility in bulls (Amann et al., 2000).

The bovine epididymal sperm post-thaw samples used in this experiment had 14.2% retained cytoplasmic droplets, down from 34% at prefreeze. This decrease in percent retained cytoplasmic droplets may actually aid in IVF fertilization, however this

does not agree with the IVF rates reported in the IVF Experiment in Chapter 5. For both 24- and 48-hour cool storage-time treatment groups, the fertilization rates with cryopreserved bovine epididymal sperm were very low (26.1 and 22.6% for the 24- and the 48-hour treatment groups, respectively).

In the present study, no significant difference was found for mean PPM values between EYG and MG cryoprotectants. A significant difference in mean PIA values between EYG and MG cryoprotectants for the preservation of bovine epididymal sperm first appear in the 24-hour cool storage-time treatment group at 0 hour and 3 hours post-thaw. A significant difference was also noted in the 48-hour treatment group at 3 hours post-thaw. In both cases the MG medium was better than the EYG medium at protecting the integrity of the epididymal sperm membranes. Karabinus et al. (1991) also found that PIA for ejaculated bull sperm was better maintained when cryopreserved in MG medium but that PPM was better maintained by EYG. The finding in our study on the bull, that MG medium was better than EYG medium, was in contrast to that of Schmid et al. (1997), who reported on cryodiluents for Greater kudu epididymal sperm. Schmid et al. (1997) reported that the overall most effective medium was Biladyl with 7% glycerol for the cryopreservation of Greater kudu epididymal sperm. Biladyl medium is an egg-yolk based, similar to the 7 EYG used for our experiment, and not milk-based medium. Lonergan et al. (1994) also reported on the efficacy of Biladyl compared with a milk-based medium to cryopreserve bovine sperm to be used for IVF. The Biladyl medium preserved sperm produced a higher cleavage rate after in vitro insemination but had a similar blastocyst development rate as the sperm cryopreserved in milk-based medium.

The PPM of caprine epididymal sperm was significantly different among 24- and 48- through 96-hour cool storage-time treatment groups at the prefreeze time measurement.

This pattern was similar to that noted in a previous goat epididymal sperm experiment (Chapter 3), as well as, prefreeze values reported for Sika deer epididymal sperm maintained in cool storage at 4°C (Hishinuma et al., 2003).

In the present study, a significant difference was also noted at prefreeze time of measurement between the 24- and 96-hour groups for mean PIA. These mean PPM and PIA prefreeze values are greater in our study than values reported for Spanish Cantabrian Chamois epididymal sperm collected <120 hours postmortem (Anel et al., 2000).

However, mean PIA values in our study were lower than the 92% viable sperm reported by Blash et al. (2000) for goat epididymal sperm harvested within 10 minutes of death. Our PIA values were also lower than those reported for epididymal sperm of Sika deer that were kept in cool storage for up to 7 days postmortem (Hishinuma et al., 2003). No differences were noted for the 24- through 96-hour cool storage-time treatment groups at 0 hour or 3 hours post-thaw.

Caprine epididymal sperm previously stored at 4°C in the epididymides of testes for 24 and 48 hours postmortem and cryopreserved in EYG had a decrease in mean PPM from prefreeze to 3 hours post-thaw and were found to have a decrease from prefreeze to 0 hour post-thaw for the 72-hour treatment group. No significant difference was detected in mean PPM values over measurement time in the 96-hour cool storage-time treatment group. All four cool storage-time treatment groups (24 through 96 hours) had a significant decrease in mean PIA values from prefreeze to 0 hour post-thaw. This difference from prefreeze to post-thaw PIA values is in contrast to the maintenance of sperm membrane integrity of Sika deer epididymal sperm that had been cryopreserved in 8% EYG after cool storage for up to 24 hours (Hishinuma et al., 2003). In the latter study, there was a

decrease in PIA values of Sika deer frozen-thawed epididymal sperm after 96 hours of cool storage.

The caprine epididymal sperm cryopreserved with MG medium was significantly different for mean PPM from prefreeze to 0 hour post-thaw for the 24-hour cool storage-time treatment group. No difference was noted for mean PPM values in cool storage-time treatment groups (48, 72 or 96 hours) when assessed over measurement times. Mean PIA for the sperm samples cryopreserved in MG medium were found to have a significant difference at 24 and 48 hours cool storage-time treatment groups between prefreeze and 0 hour post-thaw. The 72-hour cool storage-time treatment group was significantly different in mean PIA values between prefreeze and 3 hours post-thaw and the 96-hour treatment group was significantly different between 0 and 3 hours post-thaw. A significant difference in mean PIA values at 3 hours post-thaw time measurements. This is indicative of latent cryoinjuries that were not detected immediately upon thawing, which in the bull is considered a good measure of sperm fertility following cryopreservation (Saacke, 1970; Fleming et al., 1976; Chandler et al., 1978).

Significant differences were found between EYG and MG cryoprotectants for mean PPM and PIA values for the buck at the 24-hour cool storage-time treatment group at 0 and 3 hours post-thaw. No differences were detected between the cryoprotective media for the 48-, 72-, or 96-hour cool storage-time treatment groups. At both 0 and 3 hours post-thaw, as with the cryopreserved bovine epididymal sperm, MG medium was better at protecting the integrity of the epididymal sperm membranes based on mean PPM and PIA values. Leboeuf et al. (2000) reported that buck ejaculated sperm also showed higher survivability when cryopreserved in EYG versus MG based media.

As previously noted in Chapter 3, there was an unusual upward trend in both PPM and PIA for cryopreserved caprine samples in both EYG and MG media. Post-thaw values at 0 and 3 hours tended to be greater from the 24-hour to the 96-hour treatment group. This increase has also been previously described in equine males (Chapter 4). One possible explanation for this increase in post-thaw survival is the natural attrition of substandard sperm cells by cool storage in the epididymis at 4°C. The longer the testicles are kept in cool storage, the longer the sperm are exposed to necrosing tissue and the process of apoptosis. As the sperm approach 96 hours of storage, it is possible that the remaining sperm cells are more viable and less cryosensitive. At this time, no literature was found to support our observations.

The mean PPM and PIA for equine epididymal sperm did not exhibit a difference among the 24-, 48-, 72- or 96-hour cool storage-time treatment groups at the prefreeze, 0-hour or 3-hours post-thaw time measurements. Mean PPM and PIA values were slightly different than results obtained in for the stallion (Chapter 4). The epididymal sperm PPM values of our experiment are greater than values reported for domestic stallions when epididymal sperm samples were collected after 24 hours postmortem at 5°C (Bruemmer et al., 2002). However, the pattern of PPM reported in our study was similar to that reported for two domestic South African stallions (Lubbe et al., 2000).

Equine epididymal sperm previously stored at 4°C in the cauda epididymidis of the testes for 24 through 72 hours postmortem and preserved in SM-EYG cryoprotectant had a significant decrease in mean PPM over time between prefreeze and 3-hour post-thaw samples. No significant difference was found over time in the 96-hour cool storage-time treatment group. A similar decrease in equine epididymal sperm cryopreserved in SM-EYG for our study from prefreeze to post-thaw was also noted for equine epididymal

sperm collected immediately after death (Tiplady et al., 2002) or 24 hours postmortem (Bruemmer et al., 2002). All four cool storage-time treatment groups (24 through 96 hours) in our study were found to have a significant decrease in mean PIA value over time from prefreeze to 0 hour post-thaw. This difference in mean PIA from prefreeze to 0 hour post-thaw is the same as that noted for both bovine and caprine cryopreserved (MG) epididymal sperm from the 24- and 48-hour cool storage-time treatment groups in this study.

In this study, the equine epididymal sperm that had been cryopreserved with EYG had a significant decrease in mean PPM values over time from prefreeze to 0 hour post-thaw for all four (24 through 96 hours) cool storage-time treatment groups. Bovine epididymal sperm samples cryopreserved in MG exhibited a decrease from prefreeze to 0 hour post-thaw for both the 24 and 48 hour cool storage-time treatment groups while caprine epididymal sperm only showed this decrease for the 72 hour group.

Mean PIA for the samples cryopreserved in EYG exhibited the same significant difference for all four cool storage-time treatment groups from prefreeze to 0 hour post-thaw. This pattern for PIA was also noted for all cool storage-time treatment groups for prefreeze to 0 hour post-thaw in both bovine and caprine epididymal sperm samples. Morris et al. (2000) reported equine epididymal sperm membrane integrity values in their 24-hour postmortem group for both prefreeze and post-thaw similar to equine epididymal sperm PIA values collected at 0 hour postmortem in our study.

A significant difference was noted between SM-EYG and EYG cryoprotectant for mean PPM values for the 24-, 48-, 72- and 96-hour cool storage-time treatment groups at 0 and 3 hours post-thaw. This difference found in PPM from epididymal sperm between the two media was not in agreement with the findings of Lubbe et al. (2000) for two stallions.

In the latter study, there was no difference in PPM values between the two cryopreservation media, which were very similar to those used for our study. The inconsistency may be due to the small number of samples used by Lubbe et al. (2000).

Unlike mean PPM values for the stallion, no significant differences were detected for mean PIA values between SM-EYG and EYG for the preservation of equine epididymal sperm in all four cool storage-time treatment groups at 0 hour and 3 hours post-thaw. The difference in mean PPM values between SM-EYG and EYG for all cool storage-time treatment groups the skim milk egg yolk plus 4% glycerol medium maintained the motility at a greater rate than the egg-yolk citrate plus 4% glycerol medium. The same trend in mean PIA values was noted. These findings suggest that the SM-EYG medium would be a better cryoprotecting agent for membrane integrity of the equine epididymal sperm.

Overall, all three domestic males (bovine, caprine and equine) assessed in this experiment were found to exhibit a preference for the milk-based over egg-yolk based medium for cryopreservation of cauda epididymal sperm. Offspring have been produced in farm animals from cryopreserved epididymal sperm from bovine ICSI (Goto et al., 1990; Goto, 1993), caprine AI (Blash et al., 2000) and equine AI (Barker and Gandier, 1954). Therefore, sperm after being stored at 4°C over storage times (24, 48, 72 and 96 hours) and cryopreserved in either media (SM-EYG or EYG), could be used to produce offspring in the future.

Conclusion

In this study, results from all three domestic male species (bovine, caprine and equine) have proven that epididymal sperm collected postmortem can be cryopreserved either by an egg-yolk or milk-based medium. With all three males, the medium that

affords more cryoprotection would be the milk-based medium or milk plus 7% glycerol for the bovine and caprine epididymal sperm and skim milk egg yolk plus 4% glycerol for the equine epididymal sperm. The number of sperm recovered intact in both tested media was not ideal, indicating a need for further experimentation. The number of epididymal sperm recovered could be used with ICSI for the production of embryos, and likely offspring for all three species. Although the technique is not efficient, ICSI has produced offspring for the sheep (Gomez et al., 1998), the horse (Cochran et al., 1998, 2000; Li et al., 2001) and the cow (Goto et al., 1990; Goto, 1993; Hamanoi et al., 1999). Future research might include adjusting the freezing curve used to cryopreserve the samples, changing the percent or components used in the cryoprotectant. Also, one should not overlook sperm concentration or changing sample volume used for freezing.

CHAPTER 7

CRYOSURVIVABILITY OF BOVINE EPIDIDYMAL SPERM STORED IN THE EPIDIDYMIDES OF TESTES AT 4°C FOR 24 AND 48 HOURS SUBJECTED TO REPEATED FREEZE-THAW CYCLES

Introduction

Previous experiments have shown that among domestic males of the equine, caprine and bovine species viable epididymal sperm can be collected up to 96 hours postmortem when the testes are maintained at 4°C (James et al., 2002; Chapters 3 through 5). To increase the value of postmortem-collected epididymal sperm, preservation methods need to be created that can further increase the utility of the sperm over time. Cryopreservation and possibly repeated freezing and thawing techniques would be extremely beneficial when applied to valuable and limited supplies of epididymal sperm.

Epididymal sperm recovered from cat (Pushett et al., 2000), dog (Stilley et al., 1999, 2000), domestic cattle, goat and horse males (Chapters 3 through 6) various cloven-hoofed African wild mammal males (Black wildebeest, blesbok, Roan antelope, gemsbok, nyala, eland and African buffalo) (Kilian et al., 2000), and in addition, stallion, zebra and rhinoceros males (Lubbe et al., 2000) can be preserved by cryopreservation with minimal loss of motility. Epididymal sperm collected after 6 hours postmortem displayed enough survivability after cryopreservation (Stilley et al., 2000; Chapters 3 through 6). No studies to date have been reported on the effects of repeated freezing and thawing procedures on epididymal sperm.

There have been occasions in which a straw of semen was mistakenly thawed before the problem was identified and could be corrected. If that error was made with a limited frozen semen supply from a valuable animal, serious economic losses could be incurred. With increased use of newer ART techniques fewer sperm can be used to

produce offspring than from a commercially prepared 0.5-ml plastic straw. In particular, a technique known as intracytoplasmic sperm injection (ICSI) requires only one sperm cell per oocyte to achieve fertilization. Furthermore, the sperm cell utilized in ICSI does not have to be motile to achieve ICSI fertilization (Goto et al., 1990; Goto, 1993; Motoishi et al., 1996). Goto et al. (1990) and Goto (1993) produced a live calving by using ICSI with frozen-thawed bovine epididymal sperm. It would be beneficial if straws of semen could be thawed and refrozen without detrimental effects on the ability of the sperm cells to fertilize an oocyte.

Researchers in other scientific fields have tested the viability and stability of various substances when subjected to a thaw-refreeze protocol. At the Mayo Clinic, DiMagno et al. (1989) tested the effect of thawing and refreezing on selected constituents of human serum. Most measurements exhibited significant variability over test cycles when thawed six times. These differences were believed to be due to laboratory variability. It was determined that the repeated thawing and refreezing did not affect the results of any tested constituents of serum (DiMagno et al., 1989). Another study (Dzik et al., 1989) conducted on refreezing blood constituents looked at the coagulation properties of fresh-frozen plasma (FFP) compared with that of refrozen samples. A decrease in properties of the refrozen samples appeared but the differences were deemed to not be clinically important. Kahn et al. (1978) also exposed red blood cells to the thaw-refreeze test. Red blood cell survival was not adversely effected by the refreeze procedure.

With mammalian embryos, it has been demonstrated that mouse embryos can survive 3 freeze-thaw cycles and still develop during culture in vitro (Vitale et al., 1997). Studies by Snabes et al. (1993) subjected 8-cell embryos to the following protocol: thaw, biopsy, refreeze, thaw, culture in vitro and transfer. Results indicate that cryopreserved

mouse embryos could successfully undergo thawing, biopsy and refreezing. Snabes et al. (1993) suggested that under certain conditions, cryopreservation might be utilized in genetic diagnosis schemes. Other researchers have found that embryos in the pronuclear stage could also survive the thaw-refreeze process (Baker et al., 1996). In a case study, Baker et al. (1996) reported a successful pregnancy after the transfer of human embryos that were frozen twice before transfer, the second freezing occurred during the pronuclear stage of development.

Refreezing sperm is not a new idea. Filseth and Vatn (1979) used refreezing as a means of predicting the viability and fertility of bull sperm. They reported that the motility of the sperm had decreased after refreezing but when the sperm was utilized for artificial insemination (AI) there was no difference in rate of return between samples frozen once and those frozen twice. Dyer et al. (1987) also reported that progressive motility decreased after refreezing but also noted that morphology of the motile sperm was normal. In another study, Leffler and Walters (1996) conducted thaw and refreeze procedures and concluded that sperm subjected to multiple freeze-thaw processes would exhibit improved motility recovery if the thawing was conducted at 37°C compared with that of room temperature. Bandularatne and Bongso (2002) also evaluated human sperm function by assessing fertilization from ICSI with hamster oocytes with human sperm after repeated freeze-thaw cycles. They reported fertilization after the third freeze-thaw cycle. These findings suggest refrozen sperm could therefore be used for ICSI.

The purpose of this study was to determine if cryopreserved epididymal sperm collected 24 and 48 hours postmortem could survive repeated thawing and refreezing procedures and continue to maintain enough acrosomal integrity to be useful for procedures, such as in vitro fertilization (IVF) or ICSI.

Materials and Methods

Experimental Design

Commercial beef bull semen samples (n=30 bulls at 2 straws each) were collected and cryopreserved with the standard protocol (Genex, Inc.) in egg-yolk citrate with 7% glycerol as the cryoprotective agent (Zaugg and Almquist, 1972). Semen samples were transferred to the Embryo Biotechnology Laboratory on the Louisiana State University campus for further analysis (Treatment A). Cryopreserved samples of bovine epididymal sperm (beef type bulls) (Treatment B), were collected during previous experiments after testes were maintained in cool storage of 4°C for 24 or 48 hours postmortem. Only animals (n=30) that had 2 or more straws stored in liquid nitrogen (LN₂) for both cool storage-time treatment group (24 and 48 hours) were used for this experiment. Epididymal sperm and ejaculated sperm samples were thawed and a 0.125 ml sample of each straw was removed and exposed to sperm analysis at 0 and 3 hours post-thaw. Upon removal of 0.125 ml of the cryopreserved sample, the straws were refrozen by a standard procedure for further analysis on consecutive thaw cycles. Each analysis included the percent progressively motile (PPM) and percent intact acrosome (PIA).

Experimental Procedure

Sperm TL medium (Parrish et al., 1985) was prepared with the addition of 0.006 g/ml of bovine serum albumin (Sigma, St. Louis, MO), 1mM sodium pyruvate and 25 µg/ml of gentamicin (GibcoBRL, Grand Island, NY). Sperm TL medium was then warmed in a water bath to 39°C for use with the epididymal sperm. The cryoprotective medium utilized for preservation of bovine samples, egg-yolk citrate with 7% glycerol (EYG) (Salisbury et al., 1941) was prepared as one batch and frozen in 10-ml aliquots.

Cryopreservation medium was thawed at 4°C until liquidified and then warmed to room temperature (25°C).

Paired bovine testicles were randomly allotted to either a 24-hour or a 48-hour cool storage-time treatment group. After 24 or 48 hours of cool storage at 4°C, bovine epididymides were minced and placed into a 60-mm tissue culture dish (Becton and Dickinson & Company, Franklin Lakes, NJ) and rinsed with 3 ml of Sperm TL medium. After 5 minutes, the tissue was removed and the sperm plus Sperm TL medium was placed in a 15-ml centrifuge tube (Corning, Inc., Corning, NY). The bovine epididymal sperm specimen was centrifuged (Sorvall® Tabletop Refrigerated Centrifuge RT-6000, Sorvall® Centrifuges, Wilmington, DE) at 700 x g for 6 minutes to produce a sperm pellet. The Sperm TL medium supernatant was removed and the bovine epididymal sperm pellet was resuspended in fresh Sperm TL medium to create a suspension of 30×10^6 sperm/ml. Ten microliters was removed from the final bovine epididymal sperm plus Sperm TL medium suspension for subsequent analysis.

Sperm analysis consisted of PPM, PIA and a count of number of sperm obtained from the bovine epididymides. Analyses (PPM and PIA) were completed on a 5- μ l volume of bovine epididymal sperm suspension after the addition of 95 μ l of Sperm TL medium, using a 1:20 dilution factor. The other 5 μ l was added to 95 μ l of distilled water (a 1:20 dilution) to place on a hemacytometer (American Optical, South Bridge, MA) to obtain an accurate sperm count. Motility was assessed on the 10 μ l of 1:20 sperm solution using Sperm TL medium dilution with phase contrast optics and a 100X objective on a Nikon Labophot microscope (Nikon Instruments, Inc.). An eosin B/fast green stained bovine epididymal sperm specimen on a microscope slide was also assessed for sperm membrane and acrosomal integrity (Aalseth and Saacke, 1986). Then 20 μ l of the 1:20

sperm solution was mixed with 10 µl of eosin B/fast green stain. After co-incubating for 1 minute the eosin B/fast green mixture was pipetted on to a slide (10 µl), spread over the slide and heat fixed.

Each bovine epididymal sperm sample was analyzed by counting 200 sperm and membrane integrity category was determined. Cells were categorized as having an intact membrane, if they retained sperm cell membrane integrity and excluded the dye. Sperm that appeared pink or red were considered membrane compromised and therefore nonviable.

When motility and total number of sperm in a sample were determined the remaining sperm sample was cryopreserved (Figure 7.1). Each bovine epididymal sperm specimen was centrifuged at 700 x g and the Sperm TL medium was removed from the pellet and part A (0% glycerol) of EYG extender was added to create a 30×10^6 sperm/ml concentration. This EYG/bovine epididymal sperm dilution was cooled at 4°C for a total of 1 hour with additions of part B (14% glycerol) EYG occurring at 15-minute intervals. Each 15-minute period brought the addition of 0.125% of the total final volume. After 1 hour the sperm solution was aspirated into 0.5-ml plastic straws and relocated to the freezing chamber.

The freezing chamber (a Styrofoam[™] container) placed the straws 2 cm above the level of LN₂. At this level (2 cm) the temperature of the nitrogen vapor was -110°C. The freezing protocol for the bovine sperm required that the straws remain at this level in the vapor for 8 minutes until they reached a temperature of -100°C. At -100°C, the straws were rapidly frozen by immersion in LN₂ at -196°C. The freezing rate was -13°C/minute for both the bovine ejaculated sperm and epididymal sperm from 4°C to -100°C (Figure 7.2).

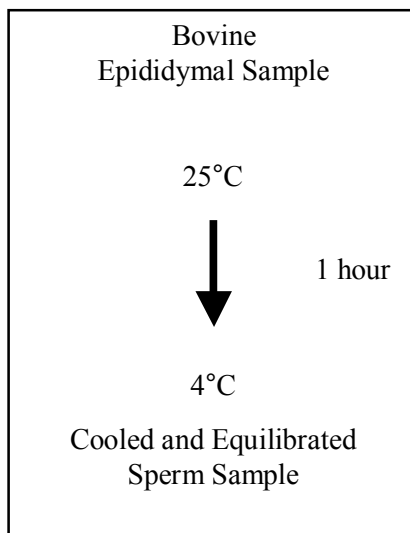


Figure 7.1. The cryopreservation equilibration protocol diagram for epididymal sperm harvested from bovine cauda epididymidis stored at 4°C for 24 and 48 hours postmortem.

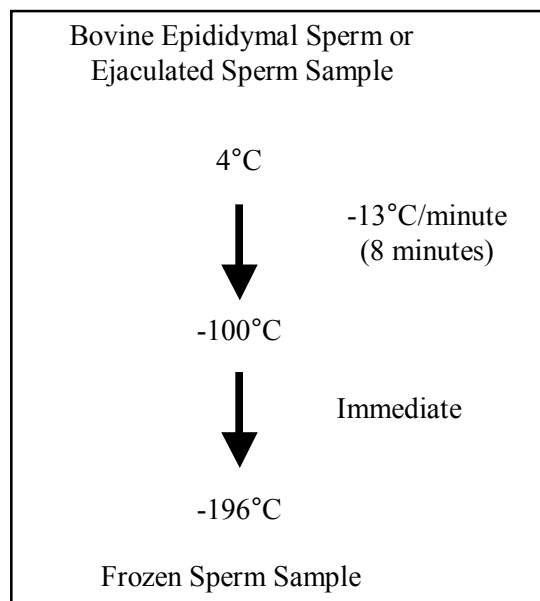


Figure 7.2. The freezing protocol diagram for ejaculated sperm and epididymal sperm harvested from bovine cauda epididymidis stored at 4°C for 24 and 48 hours postmortem.

Ejaculated sperm and epididymal sperm samples were cryopreserved for at least 24 hours before thawing for each thaw-refreeze analysis. On day of thaw analysis, Sperm TL medium was prepared and warmed to 39°C. Straws were removed from LN₂ and placed into a 39°C water bath for 1 minute. The samples (~0.125 ml) were then combined in a 5-ml conical tube with 2 ml of pre-warmed Sperm TL medium. The sperm mixture was centrifuged at 700 x g for 6 minutes and the supernatant removed from the pellet. The bovine sperm pellet was then resuspended with another 2 ml of Sperm TL medium and washed again. Processing aids in diluting the glycerol away from the epididymal and ejaculated sperm. Sperm pellets, both ejaculated and epididymal were resuspended in 500 µl of Sperm TL medium and prepared for analysis.

After removing the cryopreserved sperm sample portion to be analyzed, the remainder of the sample was immediately placed through the freezing process. Each straw was resealed and placed into storage at 4°C for 1 hour. At the end of an hour the samples were removed and placed in the freezing chamber. They were again placed 2 cm above the level of the LN₂ and allowed to cool for 8 minutes until they reached -100°C. At which time the shortened straws of sperm were plunged into the LN₂ to complete the refreezing process. Again each sample was allowed to remain frozen for at least 24 hours before another thaw/freeze cycle was attempted.

Statistical Analysis

Progressively motile and acrosomal intact sperm are given as a mean value \pm SEM for sperm parameters. Data were analyzed using Proc Mixed in SAS[®] (Version 8.0) and a repeated measures design, with a Tukey multiple comparison adjustment. This analysis determined if there was a difference among cool storage-time treatment groups (24 and 48

hours) of epididymal sperm as well as ejaculated sperm by examining the difference between the prefreeze and post-thaw PPM and PIA values for each of the freeze-thaw cycles as well as the differences found in the number of freeze-thaw cycles survived (SAS, 1996).

Results

Freeze-Thaw Cycle One

In freeze-thaw cycle 1, mean PPM at 0 hour post-thaw for bovine epididymal sperm exhibited a decline ($P=0.025$) from the 24-hour to the 48-hour group (37.8 ± 2.2 and $25\pm2.2\%$, respectively) (Figure 7.3). The mean PPM value for the bovine ejaculated sperm sample at 0 hour post-thaw of $35.4\pm1.6\%$ does not differ from either the 24- or the 48-hour epididymal sperm. No differences were found among the mean PPM values at 3 hours post-thaw for 24- or 48-hour epididymal sperm or ejaculated sperm samples (25.8 ± 2.0 , 21.4 ± 2.0 and $26.3\pm1.5\%$, respectively).

Live sperm were obtained from 0-hour post-thaw samples in freeze-thaw cycle number one as found with mean PIA values for 24- and 48-hour epididymal sperm and ejaculated sperm (42.9 ± 2.6 , 36.9 ± 2.6 and $38.6\pm1.9\%$, respectively) (Figure 7.4). Mean PIA values for 3 hours post-thaw also showed acrosomal integrity for all three groups, 24- and 48-hour epididymal sperm and ejaculated sperm with 30.2 ± 2.0 , 32.8 ± 2.0 and $27.1\pm1.5\%$, respectively. There was no difference for any of the sperm groups (24-, 48-hour epididymal sperm or ejaculated sperm) at 0 hour or 3 hours post-thaw for PIA.

Freeze-Thaw Cycle Two

Mean PPM for freeze-thaw cycle 2 at 0 hour post-thaw for bovine epididymal sperm from the 24-hour and 48-hour cool storage-time treatment groups were significantly different ($P<0.0001$) less than the ejaculated sperm group (3.9 ± 2.2 , 3.6 ± 2.2 and

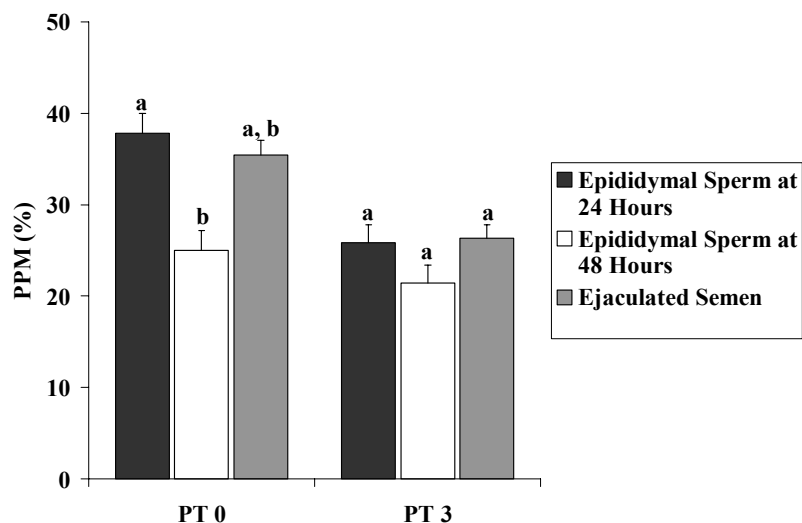


Figure 7.3. Mean (\pm SEM) percent progressively motile (PPM) sperm at 0 and 3 hours post-thaw (PT) from cryopreserved samples, frozen and thawed one time, of bovine epididymal sperm processed after 24 hours or 48 hours of storage in the cauda epididymidis of testes at 4°C or bovine ejaculated sperm processed immediately. ^{a,b}Means with different superscripts within time of measurement are significantly different ($P=0.0250$).

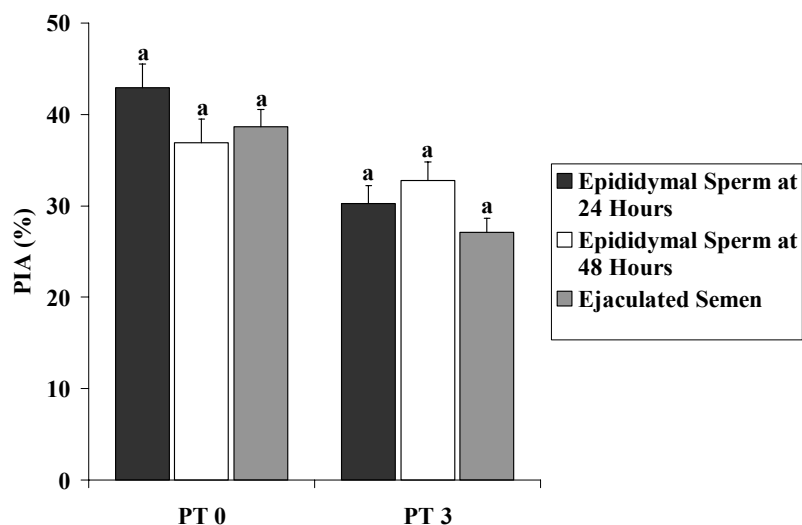


Figure 7.4. Mean (\pm SEM) percent intact acrosome (PIA) sperm at 0 and 3 hours post-thaw (PT) from cryopreserved samples, frozen and thawed one time, of bovine epididymal sperm processed after 24 hours or 48 hours of storage in the cauda epididymidis of testes at 4°C or bovine ejaculated sperm processed immediately. There were no significant differences ($P>0.05$).

23.6±1.6%, respectively) (Figure 7.5). Mean PPM values at 3 hours post-thaw for 24- and 48-hour cool storage group epididymal sperm were also significantly less than the ejaculated sperm (3.6±2.0, 3.9±2.0 and 17.7±1.5%, respectively) ($P<0.0001$).

Sperm PIA at 0 hour post-thaw in freeze-thaw cycle 2 exhibited a significant decline between 48-hour cool storage group epididymal sperm and ejaculated sperm (11.9±2.6 and 28.1±1.9%, respectively) ($P=0.0006$) (Figure 7.6). No difference was noted for 24-hour cool storage group epididymal sperm mean PIA value of 21.4±2.6% and the 48-hour cool storage group epididymal sperm or the ejaculated sperm. Similar differences were found for mean PIA values for 3 hours post-thaw as found for 0 hour post-thaw for all three groups, 24- and 48-hour epididymal and ejaculated sperm with 13.6±2.0, 6.8±2.0 and 17.7±1.5%, respectively. The difference between 48-hour epididymal and ejaculated sperm was significant at $P=0.0065$.

Freeze-Thaw Cycle Three

In the third freeze-thaw cycle mean PPM at 0 hour post-thaw for bovine epididymal sperm from 24 and 48 hours was significantly different ($P=0.0269$) from the ejaculated sperm group (0±2.2, 0±2.2 and 11.2±1.6%, respectively) (Figure 7.7). No differences were found for mean PPM values at 3 hours post-thaw for 24- or 48-hour epididymal sperm or ejaculated sperm samples (0.3±2.0, 0±2.0 and 4.6±1.5%, respectively).

At 0 hour post-thaw for freeze-thaw cycle 3 mean PIA values for 24- and 48-hour epididymal sperm and ejaculated sperm were 3.4±2.6, 6.0±2.6 and 15.6±1.9%, respectively (Figure 7.8). A difference was noted for the 24-hour epididymal sperm group mean PIA and the ejaculated sperm sample group mean PIA ($P=0.0435$). Mean PIA values for 3 hours post-thaw also show some acrosomal integrity for all three groups, 24- and 48-hour

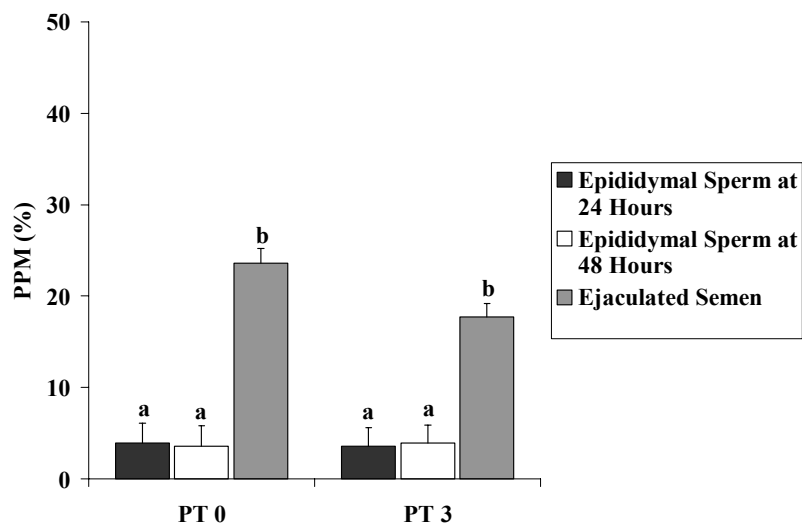


Figure 7.5. Mean (\pm SEM) percent progressively motile (PPM) sperm at 0 and 3 hours post-thaw (PT) from cryopreserved samples, frozen and thawed two times, of bovine epididymal sperm processed after 24 hours or 48 hours of storage in the cauda epididymidis of testes at 4°C or of bovine ejaculated sperm processed immediately. ^{a,b}Means with different superscripts within time of measurement are significantly different ($P < 0.0001$).

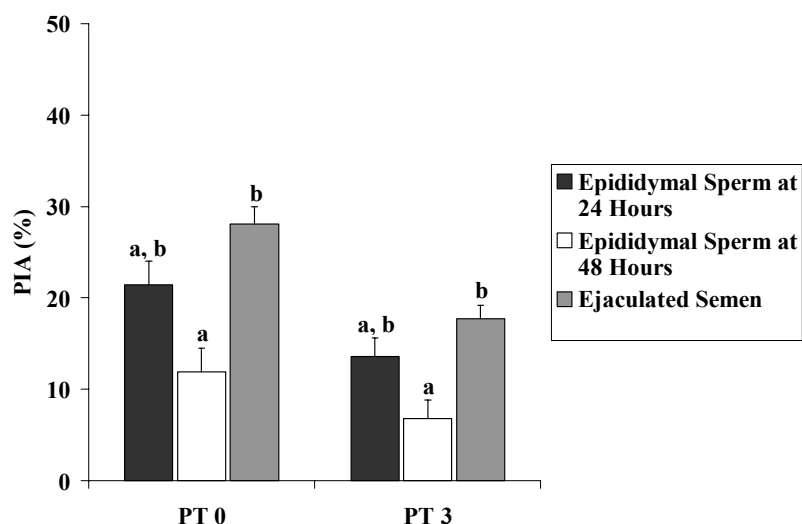


Figure 7.6. Mean (\pm SEM) percent intact acrosome (PIA) sperm at 0 and 3 hours post-thaw (PT) from cryopreserved samples, frozen and thawed two times, of bovine epididymal sperm processed after 24 hours or 48 hours of storage in the cauda epididymidis of testes at 4°C or of bovine ejaculated sperm processed immediately. ^{a,b}Means with different superscripts within the 0 hour post-thaw time of measurement are significantly different ($P = 0.0006$). ^{a,b}Means with different superscripts within the 3 hour post-thaw time of measurement are significantly different ($P = 0.0065$).

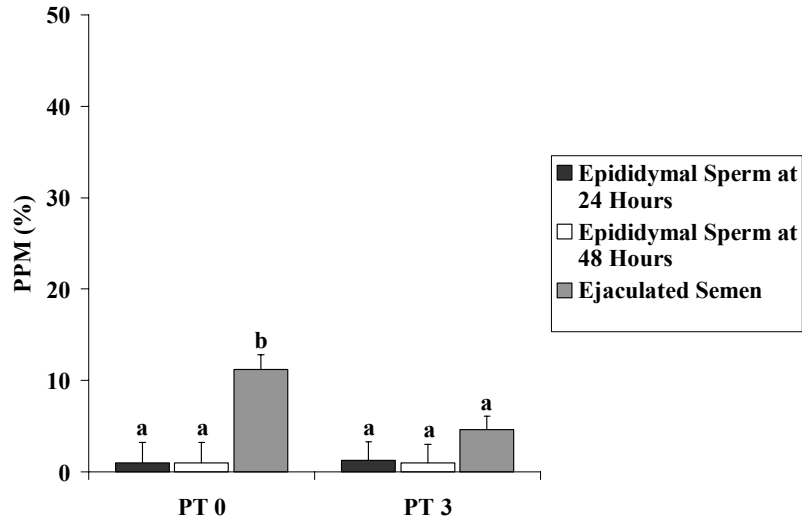


Figure 7.7. Mean (\pm SEM) percent progressively motile (PPM) sperm at 0 and 3 hours post-thaw (PT) from cryopreserved samples, frozen and thawed three times, of bovine epididymal sperm processed after 24 hours or 48 hours of storage in the cauda epididymidis of testes at 4°C or of bovine ejaculated sperm processed immediately. ^{a,b}Means with different superscripts within time of measurement are significantly different (P=0.0269).

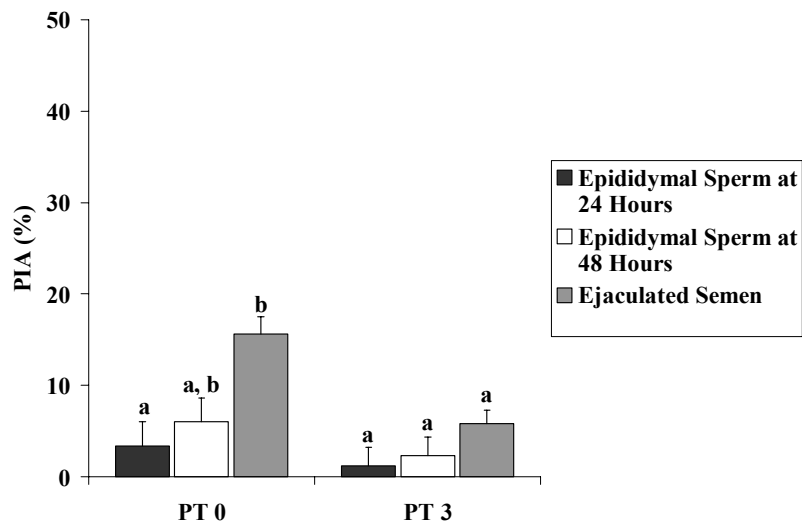


Figure 7.8. Mean (\pm SEM) percent intact acrosome (PIA) sperm at 0 and 3 hours post-thaw (PT) from cryopreserved samples, frozen and thawed three times, of bovine epididymal sperm processed after 24 hours or 48 hours of storage in the cauda epididymidis of testes at 4°C or of bovine ejaculated sperm processed immediately. ^{a,b}Means with different superscripts within time of measurement are significantly different (P=0.0435).

epididymal and ejaculated sperm with 1.2 ± 2.0 , 2.3 ± 2.0 and $5.8 \pm 1.5\%$, respectively. There were no significant differences among any of the values for any of the groups, 24-, 48-hour cool storage-time treatment group epididymal sperm or ejaculated sperm, at 3 hours post-thaw for PIA.

Freeze-Thaw Cycle Four

Motile sperm were obtained only from the ejaculated sperm group in the fourth freeze-thaw cycle (Figure 7.9). Mean PPM at 0 hour post-thaw for bovine epididymal sperm from the 24- and 48-hour cool storage-time treatment groups and ejaculated sperm groups were 0 ± 2.2 , 0 ± 2.2 and $2.0 \pm 1.6\%$, respectively, these values were not significantly different. No difference was found among the mean PPM values at 3 hours post-thaw for 24- or 48-hour epididymal sperm or ejaculated sperm samples (0 ± 2.0 , 0 ± 2.0 and $0.4 \pm 1.5\%$, respectively).

Membrane intact sperm were obtained only from 0-hour post-thaw ejaculated sperm samples in freeze-thaw cycle 4, mean PIA values for 24- and 48-hour epididymal sperm and ejaculated sperm were 0 ± 2.6 , 0 ± 2.6 and $4.8 \pm 1.9\%$, respectively (Figure 7.10). Mean PIA values for 3 hours post-thaw also show acrosomal integrity in only the ejaculated sperm with values for 24- and 48-hour epididymal and ejaculated sperm being 0 ± 2.0 , 0 ± 2.0 and $1.8 \pm 1.5\%$, respectively. There were no significant differences among any of the mean PIA values for any of the groups, 24-, 48-hour cool storage-time treatment group sperm or ejaculated sperm, at 0 hour or 3 hours post-thaw.

Comparing All Four Freeze-Thaw Cycles for the 24-Hour Epididymal Sperm Group

Results for the 24-hour bovine epididymal sperm group had mean PPM values at 0 hour post-thaw for freeze-thaw cycle 1 through 4 that resulted in a significant decline ($P < 0.0001$) from cycle 1 to 2 with no difference among freeze-thaw cycle 2 through 4

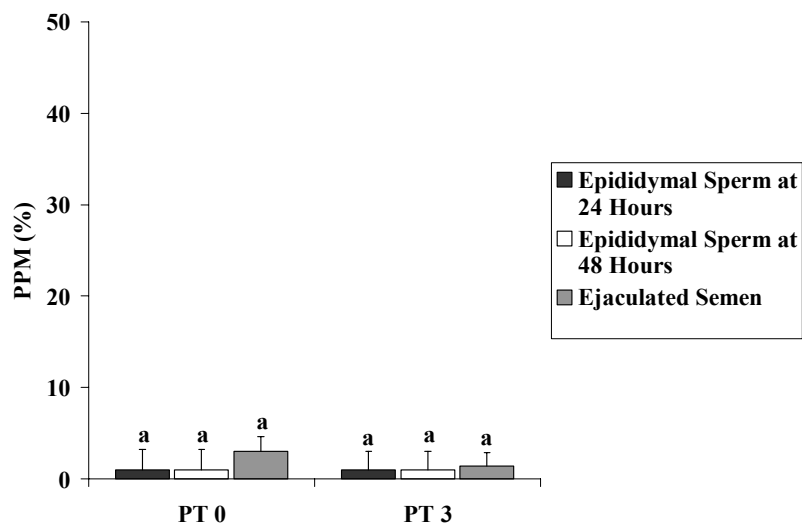


Figure 7.9. Mean (\pm SEM) percent progressively motile (PPM) sperm at 0 and 3 hours post-thaw (PT) from cryopreserved samples, frozen and thawed four times, of bovine epididymal sperm processed after 24 hours or 48 hours of storage in the cauda epididymidis of testes at 4°C or of bovine ejaculated sperm processed immediately. There were no significant differences ($P>0.05$).

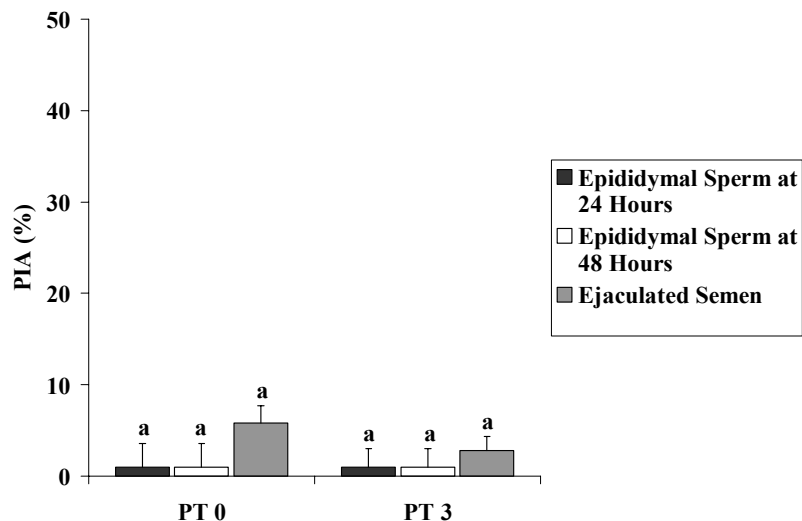


Figure 7.10. Mean (\pm SEM) percent intact acrosome (PIA) sperm at 0 and 3 hours post-thaw (PT) from cryopreserved samples, frozen and thawed four times, of bovine epididymal sperm processed after 24 hours or 48 hours of storage in the cauda epididymidis of testes at 4°C or of bovine ejaculated sperm processed immediately. There were no significant differences ($P>0.05$).

(37.8 ± 2.2 , 3.9 ± 2.2 , 0 ± 2.2 and $0 \pm 2.2\%$, respectively) (Figure 7.11). The mean PPM value at 3 hours post-thaw for freeze-thaw cycles 1 through 4 of 25.8 ± 2.0 , 3.6 ± 2.0 , 0.3 ± 2.0 and $0 \pm 2.0\%$, respectively, also exhibited the same significant decline as the values from 0 hour post-thaw. Although the decline begins between freeze-thaw cycles 1 to 2 ($P < 0.0001$) there is no significant difference found among freeze-thaw cycles number 2 through 4.

The mean PIA value for the 24-hour cool storage-time treatment group epididymal sperm at 0 hour post-thaw for freeze-thaw cycles 1 through 4 were 42.9 ± 2.6 , 21.4 ± 2.6 , 3.4 ± 2.6 and $0 \pm 2.6\%$, respectively (Figure 7.12). The significant decline began between 0 hour post-thaw mean PIA values for freeze-thaw cycle 1 to 2 ($P < 0.0001$) and from 2 to 3 ($P = 0.0009$) with freeze-thaw cycles number 3 and 4 showing no difference. Mean PIA values for 3 hours post-thaw also exhibited acrosomal integrity for the first three freeze-thaw cycles of four with 30.2 ± 2.0 , 13.6 ± 2.0 , 1.2 ± 2.0 and $0 \pm 2.0\%$, respectively. These mean PIA values exhibited the same significant differences as found at 0 hour post-thaw with freeze-thaw cycle 1 declining to 2 ($P < 0.0001$) and 2 decreasing to cycle 3 ($P \leq 0.0058$) with no difference between freeze-thaw cycles 3 and 4.

Comparing All Four Freeze-Thaw Cycles for the 48-Hour Epididymal Sperm Group

The 48-hour bovine epididymal sperm group exhibited mean PPM values at 0 hour post-thaw for freeze-thaw cycle 1 through 4 with a significant decline ($P < 0.0001$) from cycle 1 to cycle 2 but no difference among freeze-thaw cycles 2 through 4 (25 ± 2.2 , 3.6 ± 2.2 , 0 ± 2.2 and $0 \pm 2.2\%$, respectively) (Figure 7.13). The mean PPM value at 3 hours post-thaw for freeze-thaw cycles 1 through 4 of 21.4 ± 2.0 , 3.9 ± 2.0 , 0 ± 2.0 and $0 \pm 2.0\%$, respectively also showed the same significant decline as 0 hour post-thaw. The decline

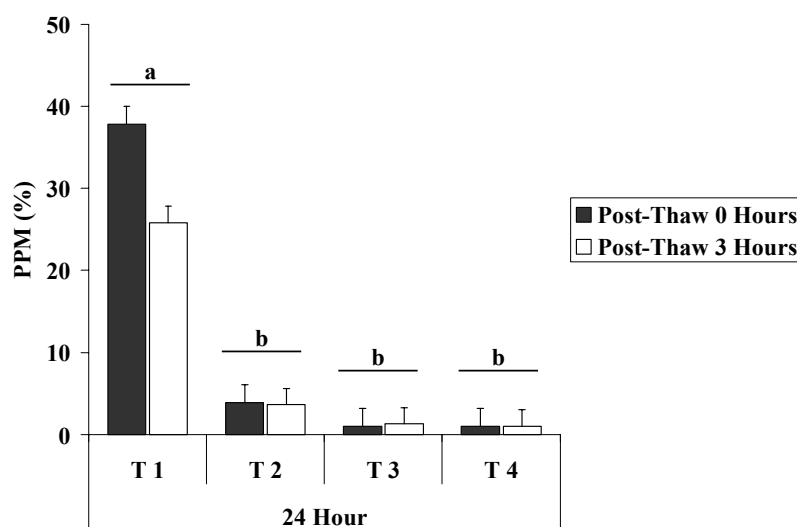


Figure 7.11. Mean (\pm SEM) percent progressively motile (PPM) sperm at 0 and 3 hours post-thaw (PT) from cryopreserved samples, frozen and thawed four times (T1 through T4), of bovine epididymal sperm processed after 24 hours of storage in the cauda epididymidis of testes at 4°C. ^{a,b}Means with different superscripts are significantly different ($P < 0.0001$).

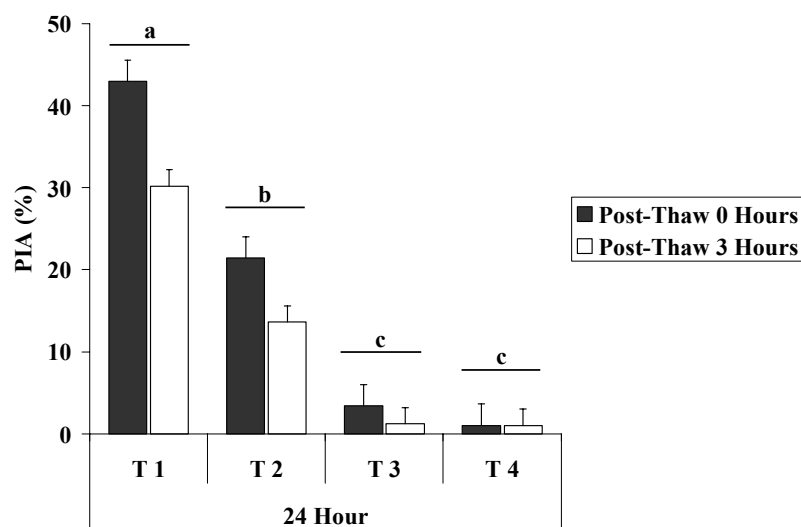


Figure 7.12. Mean (\pm SEM) percent intact acrosome (PIA) sperm at 0 and 3 hours post-thaw (PT) from cryopreserved samples, frozen and thawed four times (T1 through T4), of bovine epididymal sperm processed after 24 hours of storage in the cauda epididymidis of testes at 4°C. ^{a,b}Means with different superscripts in the 0 hour and 3 hour post-thaw time of measurement are significantly different ($P < 0.0001$). ^{b,c}Means with different superscripts in the 0 hour post-thaw time of measurement are significantly different ($P = 0.0009$). ^{b,c}Means with different superscripts in the 3 hour post-thaw time of measurement are significantly different ($P \leq 0.0058$).

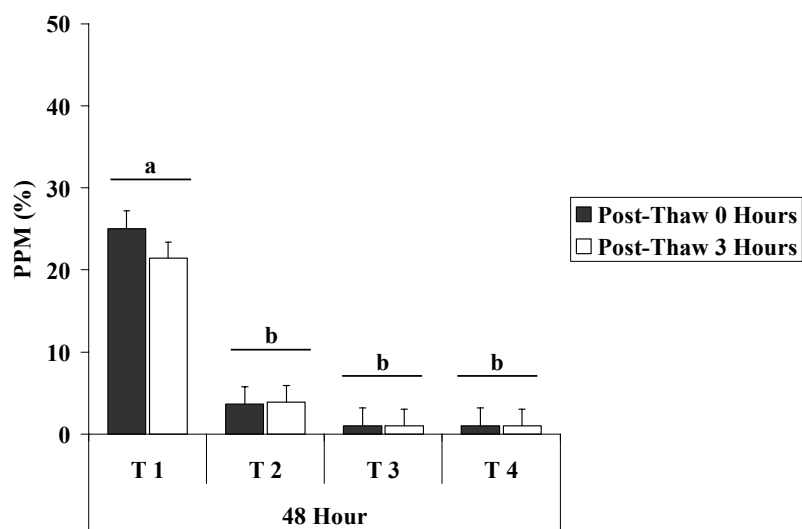


Figure 7.13. Mean (\pm SEM) percent progressively motile (PPM) sperm at 0 and 3 hours post-thaw (PT) from cryopreserved samples, frozen and thawed four times (T1 through T4), of bovine epididymal sperm processed after 48 hours of storage in the cauda epididymidis of testes at 4°C. ^{a,b}Means with different superscripts are significantly different ($P < 0.0001$).

begins between freeze-thaw cycles 1 to 2 ($P<0.0001$) with freeze-thaw cycles 2 through 4 showing no significant difference.

The mean PIA value at 0 hour post-thaw for freeze-thaw cycles 1 through 4 were 36.9 ± 2.6 , 11.9 ± 2.6 , 6.0 ± 2.6 and $0\pm2.6\%$, respectively (Figure 7.14). The decline began between freeze-thaw cycles 1 to 2 ($P<0.0001$) with freeze-thaw cycles 2 through 4 showing no difference. Mean PIA values for 3 hours post-thaw of the 48-hour cool storage-time treatment group sperm also exhibited acrosomal integrity for the first three freeze-thaw cycles of the 4 cycles with 32.8 ± 2.0 , 6.8 ± 2.0 , 2.3 ± 2.0 and $0\pm2.0\%$, respectively. These mean PIA values were also significantly different as was found at 0 hour post-thaw with freeze-thaw cycle 1 declining to 2 ($P<0.0001$) but were not significantly different among freeze-thaw cycles 2 through 4.

Comparing All Four Freeze-Thaw Cycles for the Ejaculated Sperm Group

Results from the ejaculated bovine sperm group indicate that mean PPM at 0 hour post-thaw for freeze-thaw cycles 1 through 4 exhibited a significant decline from cycle 1 to cycle 2 ($P=0.0009$), from cycle 2 to cycle 3 ($P=0.0003$) and from 3 to 4 ($P=0.0355$) with 35.4 ± 1.6 , 23.6 ± 1.6 , 11.2 ± 1.6 and $2.0\pm1.6\%$, respectively (Figure 7.15). The mean PPM value at 3 hours post-thaw for freeze-thaw cycles 1 through 4 of 26.3 ± 1.5 , 17.7 ± 1.5 , 4.6 ± 1.5 and $0.4\pm1.5\%$, respectively also showed a similar significant decline as the values from 0 hour post-thaw. The decline in mean PPM at 0 hour post-thaw for ejaculated sperm begins between freeze-thaw cycle 1 to cycle 2 ($P=0.0220$), from cycle 2 to 3 ($P<0.0001$) with freeze-thaw cycles 3 and 4 showing no difference.

The mean PIA values at 0 hour post-thaw for freeze-thaw cycles 1 through 4 were 38.6 ± 1.9 , 28.1 ± 1.9 , 15.6 ± 1.9 and $4.8\pm1.9\%$, respectively (Figure 7.16). The declines were

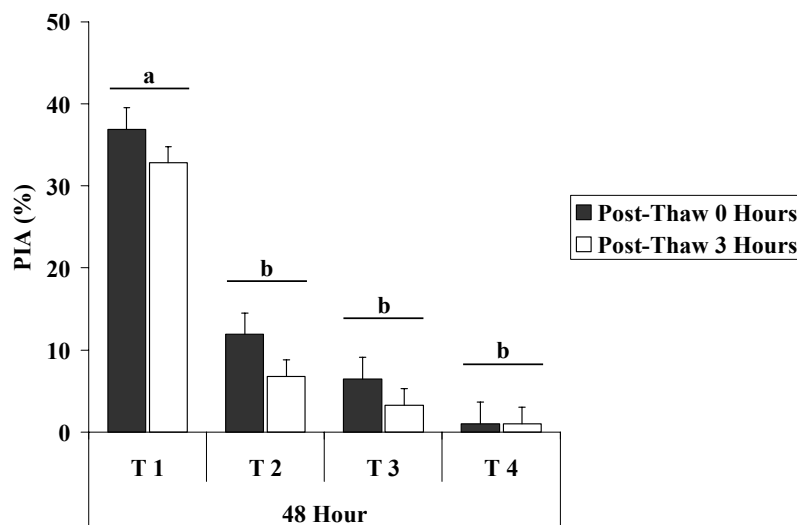


Figure 7.14. Mean (\pm SEM) percent intact acrosome (PIA) sperm at 0 and 3 hours post-thaw (PT) from cryopreserved samples, frozen and thawed four times (T1 through T4), of bovine epididymal sperm processed after 48 hours of storage in the cauda epididymidis of testes at 4°C. ^{a,b}Means with different superscripts are significantly different ($P < 0.0001$).

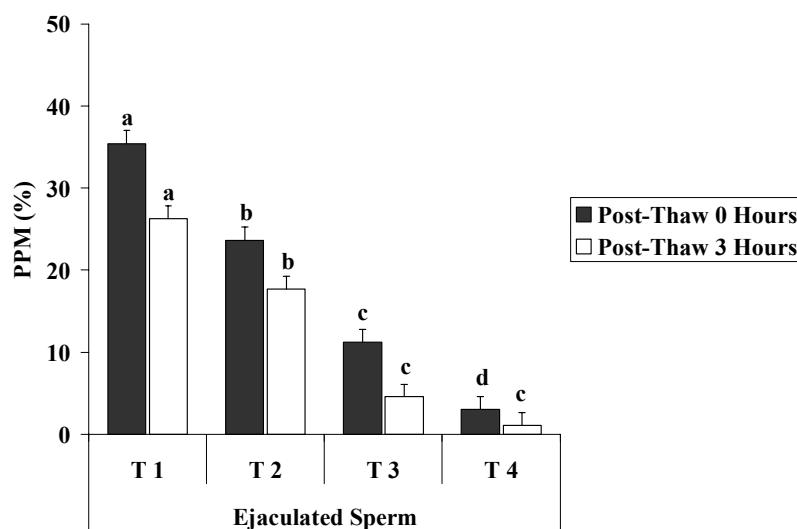


Figure 7.15. Mean (\pm SEM) percent progressively motile (PPM) sperm at 0 and 3 hours post-thaw (PT) from cryopreserved samples, frozen and thawed four times (T1 through T4), of bovine ejaculated sperm processed immediately upon collection. ^{a,b}Means with different superscripts at the 0 hour time of measurement are significantly different ($P = 0.0009$). ^{b,c}Means with different superscripts at the 0 hour time of measurement are significantly different ($P = 0.0003$). ^{c,d}Means with different superscripts at the 0 hour time of measurement are significantly different ($P = 0.0355$). ^{a,b}Means with different superscripts at the 3 hour time of measurement are significantly different ($P = 0.0220$). ^{b,c}Means with different superscripts at the 3 hour time of measurement are significantly different ($P < 0.0001$).

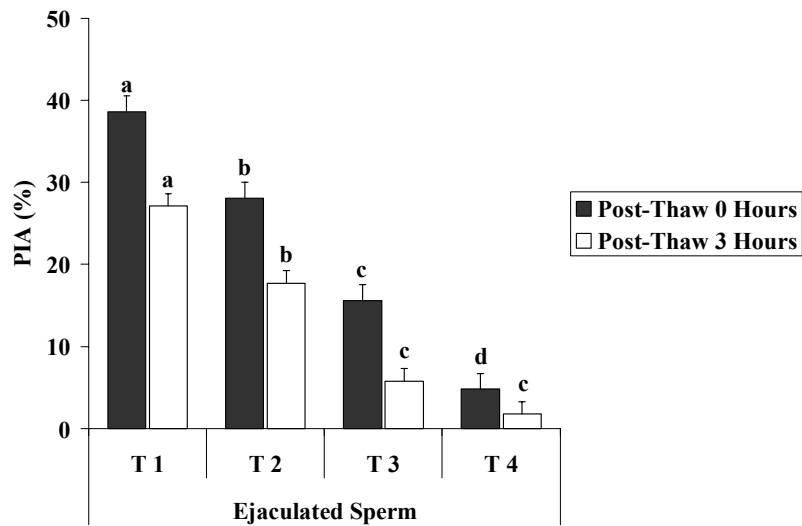


Figure 7.16. Mean (\pm SEM) percent intact acrosome (PIA) sperm at 0 and 3 hours post-thaw (PT) from cryopreserved samples, frozen and thawed four times (T1 through T4), of bovine ejaculated sperm processed immediately upon collection. ^{a,b}Means with different superscripts at the 0 hour time of measurement are significantly different ($P=0.0381$). ^{b,c}Means with different superscripts at the 0 hour time of measurement are significantly different ($P=0.0034$). ^{c,d}Means with different superscripts at the 0 hour time of measurement are significantly different ($P<0.0001$). ^{a,b}Means with different superscripts at the 3 hour time of measurement are significantly different ($P=0.0048$). ^{b,c}Means with different superscripts at the 3 hour time of measurement are significantly different ($P<0.0001$).

from freeze-thaw cycle 1 to cycle 2 ($P=0.0381$), 2 to 3 ($P=0.0034$) and 3 to 4 ($P<0.0001$). Mean PIA values for 3 hours post-thaw also were found to have at least some acrosomal integrity for all four freeze-thaw cycles with 27.1 ± 1.5 , 17.7 ± 1.5 , 5.8 ± 1.5 and $1.8\pm1.5\%$, respectively. These mean PIA values at 3 hours post-thaw for ejaculated sperm had a similar decreasing pattern through the 4 cycles as for 0 hour post-thaw with significant differences beginning with freeze-thaw cycle 1 decreasing to cycle 2 ($P=0.0048$) and cycle 2 decreasing to cycle 3 ($P<0.0001$) and no significant difference between freeze-thaw cycles 3 and 4.

The percentage of cytoplasmic droplets (CD) that were attached to the bovine epididymal sperm for both the 24- and the 48-hour cool storage-time treatment sperm groups were assessed for each freeze-thaw cycle at 0 hour post-thaw to determine if a difference would occur with increasing cryostress. Mean values are given in Table 7.1. There were no significant differences in CD rate at 0 hour post-thaw between the 24- and the 48-hour cool storage-time treatment groups. As the samples were exposed to repeated freeze-thaw cycles (one through four) no significant difference was found between the 24- and 48-hour cool storage-time treatment groups. The CD rate did not differ from freeze-thaw cycle 1 to cycle 2 but decreased from 2 to 3 ($P\leq0.0278$) with freeze-thaw cycles 3 and 4 not found to be significantly different. This decline in CD pattern was similar to the decrease in both PPM and PIA through the freeze-thaw cycles.

Discussion

Bovine epididymal sperm cryopreserved after 24 hours of storage within the epididymides of testes at 4°C can survive the cryopreservation process. These same cryopreserved bovine epididymal sperm, however, did not survive repeated freeze-thaw cycles with efficiency. The mean PPM for the 24-hour cool storage-time treatment group

Table 7.1. Mean (\pm SEM) percent of attached cytoplasmic droplets to bovine epididymal sperm processed after storage within the cauda epididymidis of testes at 4°C for 24 hours or 48 hours at prefreeze and then 0 and 3 hours post-thaw

Trt Group	Prefreeze	Freeze-Thaw Cycle			
		1	2	3	4
24 h	34.0 \pm 2.7 ^a	10.3 \pm 1.8 ^b	6.3 \pm 1.8 ^b	1.8 \pm 1.8 ^c	0 \pm 1.8 ^c
48 h	42.7 \pm 7.8 ^a	14.0 \pm 1.8 ^b	7.2 \pm 1.8 ^b	3.6 \pm 1.8 ^c	0 \pm 1.8 ^c

^{a,b,c} Values in rows with different superscripts are significantly different ($P \leq 0.0278$).

sperm at 0 hour decreased, 89% from cycle 1 to 2 and 100% from cycle 2 to cycle 3.

Determination of fertility and cryosurvival of individual bulls was assessed by PPM at 3 hours post-thaw after each freeze-thaw cycle in this experiment. This value tends to more accurately correlate with fertility of a bull than the 0-hour post-thaw PPM values (Saacke, 1970; Fleming et al., 1976; Chandler et al., 1978). After 3 hours of co-incubation with Sperm TL medium, the mean PPM values for the 24-hour group continued to show a decline and had <1% motility after the third cycle. The values decreased 85% from freeze-thaw cycles 1 and 2, 75% from cycle 2 to cycle 3 and 100% from cycle 3 to cycle 4. The epididymal sperm in this study were likely no longer useful for the production of embryos after the second freeze-thaw cycle with AI and IVF. After the second thaw, there was still 3.9% motile sperm that could be used with ICSI to produce in vitro embryos, however, the PPM was likely too low for successful IVF. Goto et al. (1990) and Goto (1993) produced a total of two calves by using ICSI with bovine epididymal sperm that was subjected to two freeze-thaw cycles to kill the sperm. In their study they cryopreserved the bovine epididymal sperm to -20°C twice to stop motility so that the sperm could be injected into the oocytes.

In this study, PIA also decreased as the freeze-thaw cycle numbers increased the decrease in PIA was 51% from freeze-thaw cycle 1 to cycle 2, 86% from cycle 2 to cycle 3

and 100% from cycle 3 to cycle 4, with no viable sperm at the end of the fourth cycle. The mean PIA pattern after 3-hours of co-incubation was similar to that of the 0-hour post-thaw decline, 53% from cycle 1 to cycle 2, 93% from cycle 2 to cycle 3 and 100% from cycle 3 to cycle 4. This decline was slower than that observed for mean PPM, indicating that after the third cycle there might be sperm that could be used for ICSI, if the sperm had not had loss of sperm viability. Garrels et al. (1998) reported that the success of ICSI in humans is not dependent upon epididymal sperm motility. They reported no significant difference between the fertilization rates of motile versus nonmotile epididymal sperm. However, they did report on the importance of using the sperm that had maintained membrane integrity. This was tested by using the hypo-osmotic swelling (HOS) test on the epididymal sperm prior to ICSI.

Therefore, in conjunction with the HOS test and ICSI these sperm could be used to create embryos and possibly offspring after the sample has undergone three freeze-thaw cycles.

Cryopreserved bovine epididymal sperm that had been previously stored in the testicle for 48 hours postmortem at 4 °C also survived the cryopreservation process. Like the 24-hour cool storage-time treatment group samples, they did not survive a repeated freeze-thaw protocol with any more efficiency. Mean PPM values at 0 hour post-thaw were found to have a decrease similar to that of the 24-hour cool storage-time treatment group. The values decreased 84% from freeze-thaw cycle 1 to cycle 2 and 100% from cycle 2 to cycle 3. At 3 hours post-thaw the decreasing PPM pattern was similar to that found with 0 hour post-thaw with 81% from cycle 1 to cycle 2 and 100% from cycle 2 to cycle 3. As with the 24-hour cool storage-time treatment group, this drastic decline in PPM at 0 hour post-thaw for the 48-hour cool storage-time treatment group would not

allow sperm from the second freeze-thaw cycle to be used with IVF to create embryos. Filseth and Vatn (1979) used repeated freeze-thaw cycles to predict the viability and fertility of bovine ejaculated sperm. They reported that sperm samples showed a decrease in PIA but no difference in the nonreturn rate of samples that were used for AI between samples frozen and thawed once or twice.

The few motile sperm found after the second cycle could possibly be used to produce embryos by ICSI. Like the 24-hour cool storage-time treatment group, the 48-hour cool storage-time treatment group had a decline in mean PIA values that extended one freeze-thaw cycle farther than the decreasing PPM values. At 0 hour post-thaw, the mean PIA values decreased 68% from cycle 1 to cycle 2, 50% from cycle 2 to cycle 3 and 100% from cycle 3 to cycle 4. The 3-hour post-thaw PIA values had the same decline of 79% from freeze-thaw cycle 1 to cycle 2, 71% from cycle 2 to cycle 3 and 100% from cycle 3 to cycle 4. Again, with no apparent motility, the samples after the third freeze-thaw cycle could only be used for the production of embryos when used in conjunction with the HOS test and possibly ICSI. Tournaye et al. (1994) reported that nonmotile epididymal sperm harvested from the distal epididymis in men could be used to achieve pregnancy. Fertilization rates with these nonmotile sperm were lower than motile sperm rates but could result in a live birth.

The results from this study suggested that cryopreserved bovine ejaculated sperm can be subjected to more than one freeze-thaw process and still maintain motility and have acrosomal integrity. The mean PPM at 0 hour and then at 3 hours post-thaw did decline, 30% from cycle 1 to cycle 2, 50% from cycle 2 to cycle 3 and 80% from cycle 3 to cycle 4, when subjected to four freeze-thaw cycles resulting in a very low progressive motility at the end of the fourth cycle. After 3 hours post-thaw the values exhibited a similar decline

of 30% from cycle 1 to cycle 2, 72% from cycle 2 to cycle 3 and 80% from cycle 3 to cycle 4 freeze-thaw cycles. Leffler and Waters (1996) reported on motility recovery of human sperm placed through two freeze-thaw cycles. Bandularatne and Bongso (2002) also evaluated human sperm function after repeated freeze-thaw cycles. They reported a decrease in PPM and PIA after each successive freeze-thaw cycle. After the third freeze-thaw cycle they reported fertilization of hamster oocytes after ICSI with the human sperm. The decline in PPM reported by Leffler and Waters (1996) and Banularatne and Bongo (2002) was similar to that observed in this current experiment for the bovine ejaculated sperm. Percent intact acrosome also decreased across the four freeze-thaw cycles, 26% from cycle 1 to cycle 2, 43% from 2 to 3 and 69% from 3 to 4, also ending with a very low PIA. Mean PIA for 3 hours post-thaw had a similar pattern to that found at 0 hour post-thaw, with a decrease of 33% from freeze-thaw cycle 1 to cycle 2, 66% from cycle 2 to cycle 3 and 66% from cycle 3 to cycle 4. Bovine ejaculated sperm maintained enough motility and acrosomal integrity to the end of the third freeze-thaw cycle to be used for IVF production of embryos. Even with the decline in PPM and PIA values there were enough sperm at the fourth cycle that could likely be used with ICSI for the production of embryos and possibly offspring.

Vitale et al. (1997) reported no difference for in vitro development of mouse embryos exposed to two or three freeze-thaw cycles at the 8- to 16-cell embryo stage. A healthy baby girl was born after the transfer of an embryo that had been frozen and thawed twice at the pronuclear stage (Baker et al., 1996). Successful repeated freeze-thaw cycles with embryos allows us to believe that repeated freeze-thaw can be applied to sperm samples with success as well.

Conclusion

Bovine sperm can be placed through more than one freeze-thaw procedure and still maintain enough acrosomal integrity to be utilized for assisted reproductive purposes. This suggests that if valuable semen was prematurely thawed it could be refrozen and saved for future use. This would not be necessarily true for epididymal sperm that had been harvested after 24 hours of cool storage in the epididymides. It could also be possible to use small amounts of sperm for IVF procedures and refreeze the rest of the sample for use at a later time. This would allow more efficient usage of bull studs by allowing 1 straw of semen to be used more than once. This could also be an important first step toward increasing the efficient use of sperm frozen in exotic endangered species that have smaller stores of sperm available.

Unfortunately, the results from the epididymal sperm in this study indicate that this technique might not be a very efficient utilization of the precious straws of cryopreserved epididymal sperm. In an effort to increase utility, freezing smaller samples for use as needed might be an alternative approach.

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APPENDIX 1

IVF MEDIA FORMULATIONS

Chemical Compound (MW)	Sigma Product Number	Fertilization Medium (mM)	TL HEPES (mM)	Sperm TL (mM)	CR1aa (mM)
NaCl (58.44)	S-5886	114	114	100	108
KCl (74.55)	P-5405	3.2	3.2	3.1	3
NaHCO ₃ (84.01)	S-8875	25	2	25	25
NaH ₂ PO ₄ H ₂ O (137.99)	S-9638	0.4	0.4	0.29	.
Na lactate (60% syrup)	L-4263	10	10	21.6	.
Ca lactate (109.1)	L-4388	.	.	.	5
CaCl ₂ ·2H ₂ O (147.02)	C-7902	2	2	2.1	.
MgCl·6H ₂ O (203.3)	M-2393	0.5	0.5	0.4	.
HEPES buffer (238.3)	H-6147	.	10	10	.
Phenol Red	P-0290	10 mg/1L	10 mg/1L	10 mg/1L	100 mg/1L
Penicillin	P-4687	100 IU/ml	100 IU/ml	.	.

Add on day of use:

Pyruvate	P-5280	0.2	0.2	1.0	0.4
Gentamicin*	15750-060	1 µl/ml	1 µl/ml	1 µl/ml	1 µl/ml
BSA**	See below	0.6% ^a	0.3% ^b	0.6% ^b	0.3% ^c
L-glutamine	G-7513	.	.	.	0.5%
FBS***	SH30070.02	.	.	.	5%
MEM amino acids	M-7145	.	.	.	1%
BME amino acids	B-6766	.	.	.	2%

Adjust as needed:

pH	.	.	7.4	7.4	7.4
Osmolarity (mOSM)	.	280-300	255-270	290-300	280-300

* Gentamicin from GibcoBRL, 50 mg/ml

** Bovine Serum Albumin (BSA):

^a Fatty-acid free (A-6003)

^b Fraction-V (A-4503)

^c FAF (A-7511)

*** Fetal Bovine Serum (FBS) Defined from HyClone®

Fertilization Medium from Bavister and Yanagimachi (1977)

TL HEPES Medium from Bavister et al. (1983)

Sperm TL Medium from Parrish et al. (1988)

CR1aa Medium from Rosenkrans et al. (1993)

APPENDIX 2

SPERM MEDIA FORMULATIONS

Chemical Compound (MW)	Sigma Product Number	7% Egg Yolk Glycerol ^a (mM)	Whole Milk Glycerol ^{a*} (mM)	Skim Milk Egg Yolk Glycerol ^b (mM)	4% Egg Yolk Glycerol ^b (mM)
Whole Milk (Kleinpeter)	.	.	93%	.	.
Nonfat Dry Skim Milk (Carnation)	.	.	.	55.8%	.
TRIZMA [®] BASE	T-1503	199.8	.	.	199.8
Citric Acid	C-8385	45	.	.	45
β-D-(-)-Fructose	F-3510	55.5	.	.	55.5
D-(+)-Glucose	G-7021	.	.	153.2	.
Na Citrate Dihydrate	S-4641	.	.	1.02	.
α-Lactose	L-2643	.	.	4.16	.
D-(+)-Raffinose	R-0250	.	.	2.52	.
Potassium citrate	P-1722	.	.	1.23	.
HEPES Buffer	H-3375	.	.	29.8	.
Dried Egg Yolk	E-0625	20%	.	4%	20%
Glycerol (Glycerin)**	G33-500	7%	7%	4%	4%
Gentamicin***	15750-060	0.5 mg/ml	0.25 mg/ml	0.5 mg/ml	0.5 mg/ml
Linco-Spectin****	UPJ61801	.	.15L/.3S mg/ml	.	.
Tylosin Tartrate	T-6134	.	.05 mg/ml	.	.

* Heat whole milk (Kleinpeter) to 95°C in boiling water over 30 to 45 minutes. Cool to 38°C at room temperature (25°C) and then to 16°C at 4°C for 2 to 3 hours. Milk has to be heated in order to inactivate lactenin in the protein fraction because of toxicity to sperm (Maxwell and Salamon, 1993; Salamon and Maxwell, 2000 and Vishwanath and Shannon, 2000).

** Glycerin from Fisher Scientific

*** Gentamicin from GibcoBRL, 50 mg/ml

**** Linco-Spectin from Pharmacia & Upjohn, 50 mg/ml lincomycin (L) and 100 mg/ml spectinomycin (S)

^a Medium used to cryopreserve bovine and caprine epididymal sperm

^b Medium used to cryopreserve equine epididymal sperm

Egg Yolk Glycerol from Salisbury et al. (1941)

Whole Milk Glycerol from Zaugg and Almquist (1972)

Skim Milk Egg Yolk Glycerol from Varner et al. (1991)

VITA

Aida “Aidita” Nioma James was born July 28, 1971, to Larry and Aida C. James in Houston, Texas. She graduated from Klein Oak High School in Spring, Texas, in 1989. Aidita earned her bachelor’s degree in animal science (Industry Option) from Texas A&M University in December of 1995. While working on her bachelor’s of science degree, she had completed an internship at Granada Equine Services in Wheelock, Texas, as an equine breeding intern with Dr. Mark Neville. She had also worked on undergraduate projects including, nutrition research with ileal-cannulated ponies and ultrasonographic cervical evaluation of mares. Aidita earned a Master of Science degree in August of 1997 under the advisement of Dr. Martha Vogelsang at Texas A&M University in physiology of reproduction with an emphasis in equine reproduction. Her thesis title was “Efficacy of Short-Term Administration of Altrenogest to Postpone Ovulation in Mares”. While working toward a Master of Science degree she was a teaching assistant in the Department of Animal Science, teaching the following courses: Introduction to Animal Science, Introduction to Equine Science, Equine Training and Management, Equestrian Technology and Equine Production and Management. She had also been involved in teaching and running portions of the Texas A&M University Horse Breeders School and The Mare/Foal Short Course.

Currently, Aidita is working toward a doctoral degree in the Department of Animal Sciences under the advisement of Dr. Robert A. Godke, Boyd Professor of Reproductive Physiology at Louisiana State University and Research Director of the LSU Embryo Biotechnology Laboratory. While at Louisiana State University she held a graduate research assistant position and worked on various projects that were being conducted by the group and by other graduate students. She was also involved in projects under the

direction of Dr. Earle Pope at the Audubon Institute for Research of Endangered Species (AICRES). The research group at the Embryo Biotechnology Laboratory of Louisiana State University also held short courses for a variety of groups and Aidita was involved in teaching various aspects of these short courses. This last year she has held a fellowship position at The A.R.T. Institute of Washington, Inc. at Walter Reed Army Medical Center in Washington, D.C. She has worked to complete her training for a laboratory director position in the human infertility field specializing in embryology.